Cardiotrophin-1 aktiviert humane periphere mononukleäre Zellen und induziert die Synthese von Tumornekrosefaktor-alpha

Dissertation

zur Erlangung des akademischen Grades
doctor medicinae (Dr. med.)

vorgelegt dem Rat der Medizinischen Fakultät
der Friedrich-Schiller-Universität Jena

von Zhenhua Wang
geboren am 04. Juli 1980 in Fujian, China

Cardiotrophin-1 Activates Human Peripheral Blood Mononuclear Cells and Induces the Synthesis of Tumor Necrosis Factor-alpha

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for the obtainment of the academic degree of
doctor medicinae (Dr. med.)

presented to the Council of the Faculty of Medicine of
the Friedrich-Schiller-Universität Jena

by Zhenhua Wang
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Tag der öffentlichen Verteidigung: 03.07.2012

Abbreviations

Akt	protein kinase B
AMV	avian myeloma leukaemia virus
Ang II	angiotensin II
ANP	atrial natriuretic peptide
AP-1	activator protein-1
ASM	airway smooth muscle
BAD	Bcl-2-associated death promoter
BNP	brain natriuretic peptide
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHF	congestive heart failure
CLC	cardiotrophin-like cytokine
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
EC	endothelial cells
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular-signal-regulated kinase
ETX	endotoxins
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GNB	gram-negative bacteria
gp130	glycoprotein 130
HRP	horseradish peroxidase

Hsp	heat shock proteins
HUVEC	human umbilical vein endothelial cells
IKK	inhibitor of kappa B kinase
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
INF	interferon
IκB	inhibitor of kappa B
JAK	janus kinase
JNK	c-Jun N-terminal kinase
LBP	LPS-binding protein
LIF	leukemia inhibitory factor
LIFR-β	leukemia inhibitory factor receptor-beta
L-NAME	NG-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
LV	left ventricle
MAPK	mitogen-activated protein kinase
MC	monocytes
MCP	monocyte chemoattractant protein
MEK	mitogen-activated protein kinase kinase
MIP	monocyte inflammatory protein
mRNA	messenger ribonucleic acid
NF-IL6	nuclear factor interleukin-6
NFκB	nuclear factor kappa B
NLS	nuclear localisation signals
NO	nitric oxide
NOS	nitric oxide synthetase
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PI3K	phosphatidylinositol 3-OH kinase
PTK	protein tyrosine kinase
RNA	ribonucleic acid

RT-PCR	reverse transcription - polymerase chain reaction
sCD14	soluble CD14 receptor
SDS	sodium dodecyl sulfate
SID	selective intestinal decontamination
SMC	smooth muscle cells
SP1	specificity protein 1
STAT	signal transducer and activator of transcription
sTNF-R	soluble tumour necrosis factor receptor
TACE	tumor necrosis factor-alpha converting enzyme
Tc cells	cytotoxic T lymphocytes
TEMED	tetramethylethylenediamine
TF	transcription factor
Th cells	helper T lymphocytes
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor-alpha

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Zusammenfassung

Bei Patienten mit chronischer Herzinsuffizienz (CHF) korreliert der Plasmaspiegel von Cardiotrophin-1 (CT-1) mit dem Schweregrad der Erkrankung, wobei die pathophysiologischen Zusammenhänge bislang unzureichend sind. Als Transmitter in der Akutphase hat CT-1 eine modellierende Rolle bei Entzündungsreaktionen. Da es über eine Cytokinantwort die Entzündung hochinflammatorisch aufrechterhält, könnte bei CHF-Patienten, die mit der Grunderkrankung assoziierte chronische Reaktion weiterhin verstärkt und prolongiert werden.

Ein wichtiger Co-Faktor bei der CHF ist der Tumornekrosefaktor α (TNF-α), der nachgewiesenermaßen selbst zu einer Herzinsuffizienz führen kann bzw. die Symptomatik der CHF weiter verschlechtert.

Entsprechend war es Ziel der vorliegenden Arbeit, die Rolle von CT1 im Hinblick auf Signalwege und Cytokinproduktion in peripheren, mononukleären Blutzellen (PBMC) zu untersuchen. Dabei sollte die Expression von TNF-α in den PBMC als zugrunde liegender Singnalweg maßgeblich erforscht werden.

Es wurden PBMC aus menschlichem Vollblut isoliert und in vitro kultiviert. Zur Messung der intrazellulären Signalproteine wurde ein Westernblott verwendet. Die mRNA der Cytokinaktivierung wurde mittels Realtime-PCR erfasst und die entsprechenden Proteine mit einem ELISA analysiert.

Es zeigte sich, dass die beiden Untereinheiten (gp130/LIFR-β) des heterodimeren Rezeptors für CT-1 auf den PBMC exprimiert werden. Über diesen Rezeptor werden CT-1 vermittelt PBMC angeregt, die extrazelluläre signalregulierende Kinase (ERK) sowie die P38 mitogenaktivierte Proteinkinase (MAKP) und damit letztlich die Expression von TNF-α zu induzieren. Somit wird durch CT-1 die Produktion von TNF-α sowohl auf mRNA- als auch auf Proteinebene in den PBMC induziert. Dabei sind Monozyten jedoch keine lymphozyteren Zellen beteiligt. Weiterhin bewirkt CT-1 eine Translokation von dem nukleären Faktor kappa B (NFκB) in den Zellkern der PBMC unter Vermittlung eines Inhibitors von κB (IκB). Nach Zugabe von Partenolid, einem Inhibitor der NFκB-Aktivierung, wird CT-1 induzierte Translokation von NFκB in den Zellkern vollständig blockiert.

Gleichzeitig führt die Gabe von Fentolide zu einer Inhibition der CT-1 induzierten Expression von TNF-α in den PBMC. Die Zugabe des MAPK-Aktivators SB203580 sowie des Inhibitors der ERK-Aktivierung PD98059 zeigten keinen Effekt auf die Expression von TNF-α.

Somit kann geschlussfolgert werden, dass die nukleäre Translokation von NFκB essentiell für die CT-1 induzierte Expression von TNF- α in den PBMC ist und die Aktivierung von NFκB unter Umgehung des MEK- und MAPK-Weges direkt erfolgt. Aufgrund der vorgestellten Experimente kann ein neuer Mechanismus der Aktivierung von PBMC postuliert werden, wobei erhöhte TNF-α Konzentrationen über den NFκB-Signalweg bei Patienten mit CHF erfolgt. Dies unterstreicht die Bedeutung von CT-1 bei der Pathophysiologie der CHF über die chronische Entzündungsreaktion hinaus. Die NFκB vermittelte TNF-α Expression in PBMC bietet möglicherweise einen neuen Therapieansatz für die Behandlung von Patienten mit chronischer Herzinsuffizienz.

Abstract

Plasma cardiotrophin-1 (CT-1) levels increase with the severity of congestive heart failure (CHF). However, the role of CT-1 in the pathophysiology of CHF remains unclear. CT-1 is a strong acute-phase mediator and might play an important role in the regulation of inflammatory process. Since immune-inflammatory activation is associated with the progression of CHF, the possibility exits that CT-1 might play a role in modulating the immune response and cytokine activation in CHF, which in turn might contribute to the deterioration and progression of CHF.

The purpose of this study is to elucidate the role of CT-1 on the activation (including signaling pathway and cytokines production) of peripheral blood mononuclear cells (PBMC), and further evaluate its effects on the expression of TNF-α in PBMC and reveal the underlying signaling pathways involved in this process.

PBMC were isolated and purified from human whole blood, and cultured in vitro. Intracellular signaling proteins were determined by Western blotting, nuclear translocation of transcription factors was evaluated by electrophoretic mobility shift assay, mRNA level of cytokines was measured by real-time PCR, and the protein production of cytokines was assessed by ELISA and flow cytometry.

This study showed that both subunits for forming the heterodimer receptor of gp130/LIFR-β for CT-1 action were expressed on PBMC. Via this receptor, CT-1 activated PBMC by evoking extracellular-signal-regulated kinase (ERK) and P38 mitogen-activated protein kinase (MAKP) signaling pathways, and promoting TNF-α expression. CT-1 significantly induced TNF-α production at both mRNA and protein levels in PBMC. Moreover, it has been shown that the subpopulation of monocytes but not lymphocytes were responsible for this effect. In addition CT-1 can induce nuclear factor kappa B (NFκB) translocation into the nucleus in PBMC, which was mediated by inhibitor of kappa B (IκB) degradation. Parthenolide (an inhibitor of NFκB activation) interrupted nuclear translocation of NFκB and completely inhibited the CT-1-induced TNF-α expression in PBMC, whereas SB203580 (an inhibitor of P38 MAPK activation) and PD98059 (an

inhibitor of ERK activation) had no effects on them. These results indicate that NFκB nuclear translocation is essential for CT-1-induced TNF-α expression in PBMC and that CT-1 might activate NFkB via Raf-1 directly as ‘‘skip’’ activation sequences but not via Raf-1/MEK/MAPKs pathway.

These observations offer a new mechanism of activated PBMC, increased serum TNF-α concentration and active NFkB signaling pathway in CHF, which have significant implications for revealing a new mechanism of immune activation in CHF. Interestingly, in the present study LPS was not needed for the induction of TNF-α expression in PBMC. Moreover, this study may also elucidate a potential role of CT-1 in the pathophysiology of CHF and illustrate that heart is not only just a target of the inflammatory state in heart failure, but also play a role in the activation of inflammatory response by secreting CT-1 into the peripheral circulation, at least in part as a cause for initiating or promoting the inflammatory response in heart failure. In the light of these results, modulating CT-1 may be an interesting pharmacological target in the treatment of CHF.

1 Introduction

1.1 Cardiotrophin-1

Cardiotrophin-1 (CT-1), a 201 amino acid protein, was originally isolated in 1995 for its ability to induce a hypertrophic response in neonatal cardiac myocytes (Pennica, King et al. 1995). Amino acid sequence similarity as well as structural homology with the interleukin (IL) -6 family indicated that CT-1 is a member of this family, which includes IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin-like cytokine (CLC), and IL-11 (Pennica, King et al. 1995). These cytokines mediate a pleiotropic set of growth and differentiation activities through an unique receptor system, consisting of the non-signaling α-receptors (IL-6Rα, IL-11Rα, and CNTFRα, where R refers to receptor) and the signal transducing receptors (glycoprotein 130 (gp130), LIFR, and OSMR). The latter associates with janus kinase (JAK) and become tyrosine phosphorylated in response to cytokine stimulation. Each of the IL-6-type cytokines is characterized by a certain profile of receptor recruitment that in all cases involves at least one molecule of gp130. Transcripts of gp130 messenger ribonucleic acid (mRNA) are found throughout murine tissues (Saito, Yoshida et al. 1992), and this could explain the pleiotropic activities of IL-6 family cytokines, including CT-1 (Latchman 2000).

IL-6, IL-11 and CNTF first bind specifically to their respective α-receptor subunits. Here, only the complex of cytokine and α-receptor efficiently recruits the signaling receptor subunits. Also, an α-receptor subunit has been postulated for CT-1 (Robledo, Fourcin et al. 1997), but since this putative receptor protein has not been cloned yet its existence is questionable. IL-6 and IL-11 are the only IL-6-type cytokines that signal via gp130 homodimers. The remaining IL-6 type cytokines signal via heterodimers of either gp130 and the LIFR (LIF, CNTF, CT-1 and CLC) or gp130 and the OSMR (OSM). Human OSM has the exceptional capability to recruit two different receptor complexes. It forms both LIFR-gp130 and OSMR-gp130 heterodimers. LIF and OSM directly engage their signaling receptor subunits without requirement for additional α-receptor subunits (Fig. 1) (Heinrich, Behrmann et al. 1998; Senaldi, Varnum et al. 1999). gp130 and

leukemia inhibitory factor receptor subunit beta (LIFR-β) is required for CT-1 signaling, because a monoclonal anti-gp130 antibody or LIFR-β antagonist completely inhibits c-fos induction by CT-1. Upon stimulation with CT-1, both gp130 and the LIFR-β are tyrosine-phosphorylated, providing further evidence that CT-1 signals through the gp130/LIFR-β heterodimer (Wollert, Taga et al. 1996).

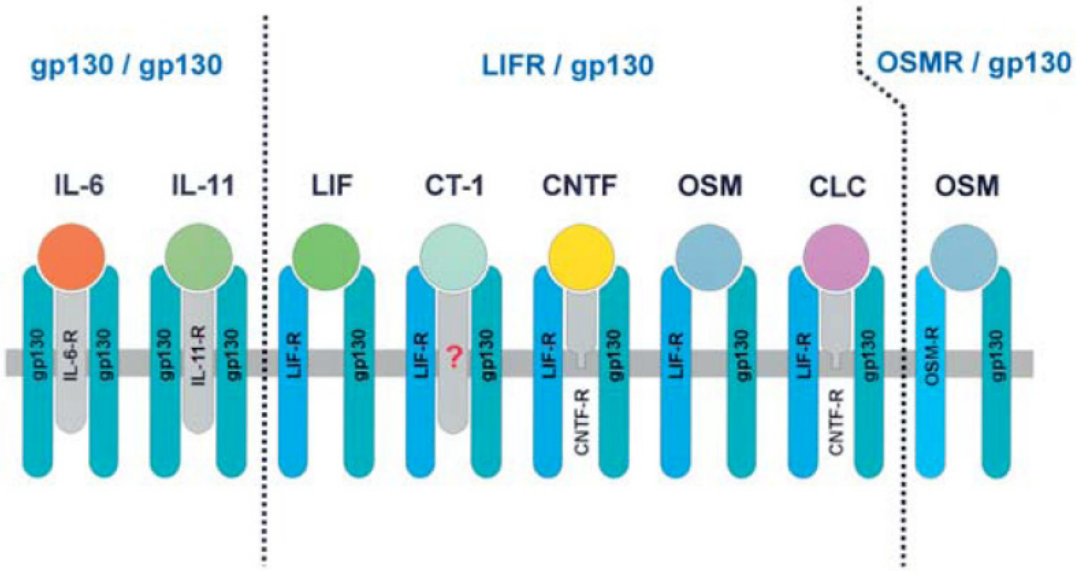


Figure 1. **Receptor complexes of IL-6-type cytokines**. IL-6-type cytokine receptor complexes signal through different combinations of the signaling receptor subunits gp130, LIFR and OSMR, with gp130 being used by all the family members (Heinrich, Behrmann et al. 2003).

The signaling pathway downstream from gp130 is reported to consist of, at least, three distinct pathways: 1) the JAK/ signal transducer and activator of transcription (STAT) pathway, 2) the mitogenactivated protein kinase (MAPK) pathway, including the extracellular- signal-regulated kinase (ERK) -1/2, P38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathway (Heinrich, Behrmann et al. 1998; Zauberman, Zipori et al. 1999; Wang, Robledo et al. 2000; Bode, Ludwig et al. 2001), 3) the phosphatidylinositol 3-OH kinase (PI3K) / protein kinase B (Akt) pathway (Kuwahara, Saito et al. 2000). Figure 2 shows the so far known signaling pathways of CT-1: Activation of the JAK/STAT pathway causes tyrosine phosphorylation of the STAT3 factor

(Yamauchi- Takihara 2002), resulting in its dimerization and transport to the nucleus where it activates its target genes (Wegenka, Buschmann et al. 1993; Akira, Nishio et al. 1994). The MAPK pathway is directly responsible for the phosphorylation and activation of nuclear factor interleukin-6 (NF-IL6), a transcription factor involved in cytokine signal transduction (Nakajima, Kinoshita et al. 1993). Finally, activation of the PI3K pathway causes the phosphorylation of Akt and the pro-apoptotic gene Bcl-2-associated death promoter (BAD) (Kuwahara, Saito et al. 2000).

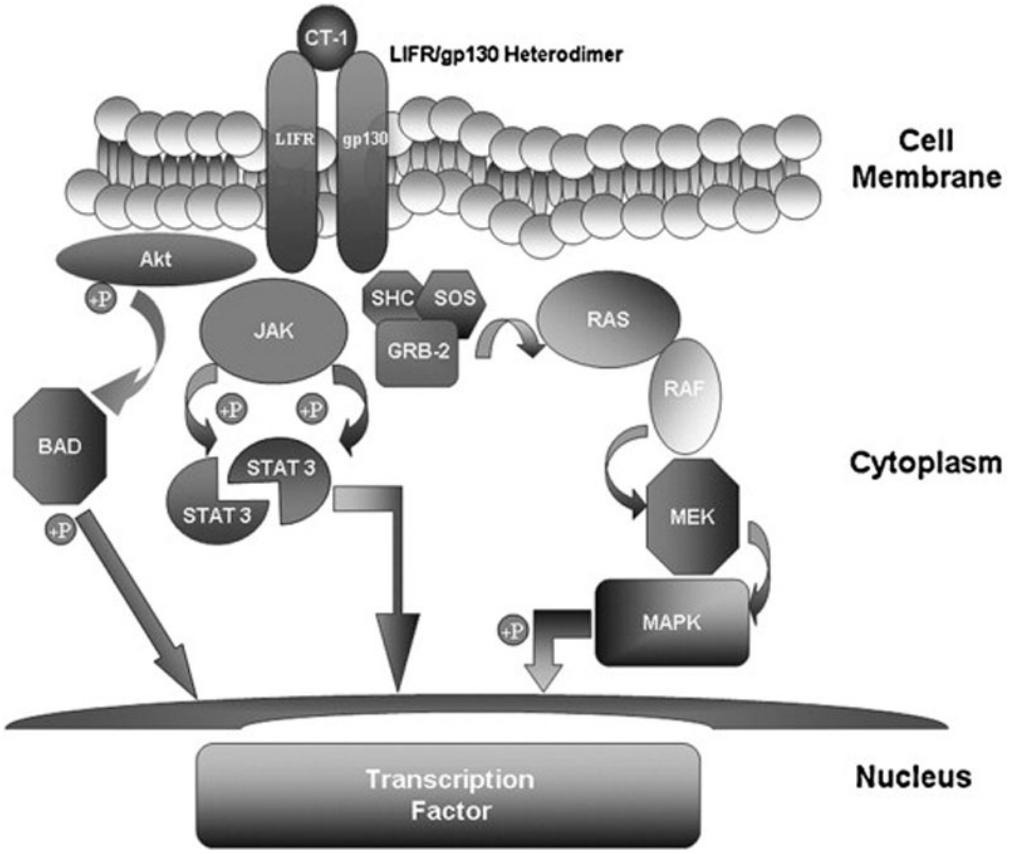


Figure 2. **Schematic structure of CT-1 receptor, composed by the LIFR/gp130 heterodimer, and the intracellular pathways activated by CT-1** (Calabrò, Limongelli et al. 2009)**.**

1.2 Expression and effects of CT-1

Many scientific works have shown that CT-1 has different effects on a variety of tissues, such as growth of the liver, kidney, and spleen; atrophy of the thymus;

increase in platelet and red blood cells count (Jin, Yang et al. 1996); defence of the liver against proapoptotic stimuli (Marques, Belza et al. 2007); increase in airway smooth muscle (ASM) mass; reduction in isoprotenerol-induced ASM relaxation (Zheng, Zhou et al. 2004); activation of JAK/STAT and MAPK pathways in both preadipocytes and adipocytes; reduction in insulin stimulated glucose uptake (Zvonic, Hogan et al. 2004); support of survival of damaged motoneurones (Oppenheim, Wiese et al. 2001; Forger, Prevette et al. 2003). CT-1 can act not only by paracrine, but also autocrine mechanisms. Transcripts of CT-1 mRNA have been detected in a variety of both neonatal and adult tissues (Pennica, Swanson et al. 1996; Sheng, Pennica et al. 1996). In the adult organism, CT-1 is produced predominantly in the heart, where it is synthesized by both cardiomyocytes and non-cardiomyocytes and where it is secreted via the coronary sinus into the peripheral circulation (Asai, Saito et al. 2000). As demonstrated by several in vivo and in vitro experimental conditions, CT-1 has several different effects, providing myocardial protection, predisposing the heart to pathological conditions, and producing haemodynamic and endocrine effects (Latchman 1999).

1.2.1 Effects on the heart

Cardiac muscle cell survival plays a critical role in maintaining the normal function of the heart. Adult cardiac muscle cells are thought to be terminally differentiated. Because they have lost their proliferative capacity, an irreversible heart injury might result in scarring and an eventual decrease in global cardiac function.

Importantly, CT-1 has been shown to be capable of promoting both the proliferation and the survival of either embryonic or neonatal cardiac myocytes at subnanomolar concentrations (Sheng, Pennica et al. 1996). Moreover, pre-treatment with CT-1 (1 ng/ml) has been shown to be able to protect cultured neonatal cardiac myocytes against subsequent exposure to either elevated temperature (heat shock) or ischemia/hypoxia (Stephanou, Brar et al. 1998). These effects were associated with the ability of CT-1 to induce enhanced levels of the heat shock proteins (Hsp) 70 and Hsp90 (Stephanou, Brar et al. 1998),

whose over-expression has been shown to protect cardiac myocytes against both thermal and ischemic stress (Latchman 2001; Liu, He et al. 2007; Jiao, Garg et al. 2008). Recent studies (Brar, Stephanou et al. 2001; Liao, Brar et al. 2002; Lopez, Diez et al. 2005) have demonstrated, in both neonatal and adult cardiac cells, the cytoprotective effects of CT-1 against ischemia, when added both prior to and after the hypoxic stimulus, and non-ischemic death stimuli, such as angiotensin II (Ang II) and hydrogen peroxide (H_2O_2).

Although CT-1 is expressed in the normal developing and adult heart, it was first isolated as a factor capable of inducing cardiac myocyte hypertrophy that is one of the most important adaptive responses of the heart and central feature of many cardiac diseases. This process, initially compensatory, causes a pathological transition from hypertrophy to dilation, which results in cardiac muscle failure at the latest stage. The original report (Pennica, King et al. 1995) showed that CT-1 was a dose-dependent potent inducer of myocardial hypertrophy, with activity being detected at concentrations of 0.1 nM or lower, and that it was more potent than other members of the IL-6 family in terms of inducing hypertrophy. In subsequent in vitro studies (Wollert, Taga et al. 1996), it was observed that the hypertrophy induced by CT-1 was distinct from that induced by α-adrenergic stimulation, both in terms of cell morphology and gene expression pattern. Stimulation with CT-1 leads to an increase in cardiac cell size that is caused by an increase in cell length without a significant change in cell width. CT-1-stimulated cells show the assembly of sarcomeric units in series (eccentric hypertrophy) rather than in parallel (concentric hypertrophy), as is observed with α-adrenergic stimulation. However, these studies indicate that CT-1 does not affect skeletal α-actin or myosin light chain-2v expression. Hypertrophic effects of CT-1 on the heart, were also confirmed in in vivo experiments (Jin, Yang et al. 1996). In fact chronic administration of CT-1 to mice (0.5-2.0 μg by intraperitoneal injection, twice a day for 14 days) induced a dose-dependent increase in both heart weight to body weight ratio, and ventricular weight to body weight ratio.

Moreover, CT-1 and its receptor complex (gp130/LIFR-β) are also present in adult canine cardiac fibroblasts, and CT-1, dose dependently, stimulates deoxyribonucleic acid (DNA) and collagen synthesis with the accumulation of

cells in the S phase (Tsuruda, Jougasaki et al. 2002; Freed, Borowiec et al. 2003). The primary function of adult cardiac myofibroblasts is to synthesize fibrillar collagens to maintain the integrity of the cardiac matrix. In addition, cardiac myofibroblasts play an important role in the progression of ventricular remodelling.

1.2.2 Haemodynamic effects of CT-1

Besides promoting cardiac growth and development, CT-1 also has effects on haemodynamics and cardiovascular functions. It was shown that intravenous administration of CT-1 (4 to 100 µg/kg) to rats caused dose-dependent systemic hypotension (Jin, Yang et al. 1998). Depressor response to CT-1 coincided with a reduction in systemic vascular resistance and resulted in a significant elevation in cardiac output associated with a concomitant increase in heart rate. This indicates that the hypotension is caused by vasodilatation. Moreover, both the depressor effect of CT-1 on blood pressure and the tachycardic effect were significantly reduced by treatment with a nitric oxide synthase inhibitor (NG-nitro-L-arginine methyl ester, L-NAME), suggesting that the haemodynamic effects of CT-1 may be mediated by nitric oxide (NO). In addition, CT-1 administration did not result in a significant alteration in left ventricular (LV) maximal dP/dt (rate of rise of left ventricular pressure), a valid index of ventricular performance, suggesting that acute administration of CT-1 does not have a significant effect on ventricular contractility. On the contrary, long-term exposure to CT-1, as observed in human heart failure, induces contractile dysfunction in heart tissues reconstituted from neonatal rat cardiac myocytes (Zolk, Engmann et al. 2005).

1.2.3 Endocrine effects of CT-1

As previously reported, CT-1 also affects endocrine function. It stimulates atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) secretion, probably with a different mechanism to G protein-coupled receptor agonists that are able

to increase the loading condition in vivo. The biological significance of increased expression of ANP and BNP genes by CT-1 is not clear at present. Although ANP and BNP have hypotensive properties in vivo, it is unlikely that ANP and BNP are responsible for the decrease in blood pressure caused by CT-1 infusion, because the hypotensive effect of ANP and BNP is not blocked by L-NAME (Hamanaka, Saito et al. 2000).

1.3 CT-1 and congestive heart failure

Due to its different effects on the heart, CT-1 is considered not only as a marker of disease, but also it can be involved in pathological changes typical of congestive heart failure (CHF). CHF is a condition in which the heart's function as a pump to deliver oxygen rich blood to the body is inadequate to meet the body's needs. It is well known that neuro-humoral factors including cytokines are activated and play an important role in the pathophysiology of CHF. The mechanism of cyclic mechanical stretch-induced myocyte hypertrophy has been examined in isolated cultures of ventricular myocytes and cardiac nonmyocytes (interstitial or perivascular cells) that highlighted the possible hypertrophic effect of the interaction between cardiac myocytes and nonmyocytes (Harada, Saito et al. 1997). Both atrial and ventricular CT-1 gene expressions are increased in experimental congestive heart failure (Jougasaki, Tachibana et al. 2000). In a subsequent study (Pemberton, Raudsepp et al. 2005), it was demonstrated that ventricular stretch stimulates CT-1 secretion.

Plasma CT-1 levels increase with the severity of CHF. Moreover, CT-1 concentrations are significantly higher in explanted hearts from patients with end-stage heart failure than in donor hearts (Zolk, Ng et al. 2002). Plasma CT-1 levels correlate with left ventricle mass index in patients with dilated cardiomyopathy (DCM) (Tsutamoto, Wada et al. 2001), which is characterized by volume overload, suggesting that CT-1 plays a role in left ventricular remodelling and/or left ventricular hypertrophy. In addition, evidence has also been presented that ventricular CT-1 gene activation preceded BNP gene activation in CHF (Jougasaki, Leskinen et al. 2003). In early left ventricular

dysfunction, the ventricular CT-1 gene expression was augmented, while the ventricular BNP gene expression was not increased. These findings raise the possibility that CT-1 is a biomarker for detecting early ventricular dysfunction in CHF as compared to BNP, which is a marker for overt CHF. Moreover, the additional prognostic value of CT-1 alone or combined with BNP has been reported in patients with CHF (Tsutamoto, Asai et al. 2007).

1.4 Immune activaion in CHF

1.4.1 Inflammation and CHF

Heart failure involves changes in several homeostatic systems, so that the syndrome may be seen as a progressive multiorgan disorder which, once originated in the heart, spreads and affects many other extracardiac sites (Mann 2002; Torre-Amione 2005). These pathophysiological processes include metabolic pathways which, although distinct, are interlinked and interact with each other, thus contributing to perpetuate and promote heart failure and cardiac remodeling, skeletal muscle cachexia, and endothelial dysfunction that characterize the most advanced forms of the disease. Even in the milder and more incipient forms of heart failure, these changes are already present and have been recently evaluated as potential markers for an early diagnosis and can, moreover, be useful as indicators of risk and prognosis (Torre-Amione 2005).

Immune and inflammatory changes in CHF have been recognized and evaluated with increasing interest in the past years. This results mainly from the reproduction of the changes that these mediators can produce in experimental models, mimicking phenotypes and different clinical patterns of the heart failure syndrome, notably in the cellular and subcellular processes associated with remodeling (Mann 2002; Torre-Amione 2005).

By extrapolating these experimental evidences to clinical studies, high levels of cytokines such as tumor necrosis factor-alpha (TNF-α), IL1 and IL6 in the circulation and in the cardiac muscle of individuals with CHF have been observed to bring important prognostic information, so that these cytokines have

been implicated in the mechanisms of CHF progression (Levine, Kalman et al. 1990; Ferrari, Bachetti et al. 1995; Torre-Amione, Kapadia et al. 1995; Torre-Amione, Kapadia et al. 1996; Hasper, Hummel et al. 1998; Adamopoulos, Parissis et al. 2001; Mann 2002; Torre-Amione 2005). These studies have generated an inexorable accumulation of evidence pointing to a progressive and repetitive state of immune-inflammatory activation associated with the progression of ventricular dysfunction, with an intense release and activation of cytokines, complement, autoantibodies, adhesion molecules and other substances in the bloodstream (Mann 2002; Torre-Amione 2005).

1.4.2 Pathways for immune activation in CHF

There are at least three well characterized pathways for immune activation in heart failure: ① immune activation by direct antigenic stimulation, as is seen in myocardial virus infection; ② secondary to cardiac injury whereby the exposure 'new antigens' in myocardium is capable of triggering an immune response against the heart; ③ cytokine release by cardiac cells, both myocytes and nonmyocytes, and non-cardiac cells in response to hemodynamic deterioration.

1.4.2.1 Immune activation by direct antigenic stimulation

Immune activation by direct antigenic stimulation is best seen in two respective clinical settings: in patients with acute myocarditis or in patients with acute cardiac allograft rejection. In patients with acute myocarditis, the cardiotrophic virus infects the cardiac cell and triggers a variety of immune mechanisms that leads to the accumulation of mononuclear, B and T cells. These cells are capable of producing inflammatory cytokines and release toxic oxygen species that may affect cardiac function. In addition, activated B cells also produce antibodies that may be of pathological importance. This immune activation may repress cardiac function further inducing a vicious circle. Similarly, the myocardium of a heart transplant recipient that undergoes rejection is under direct attack by immune cells. Histological evaluation of endomyocardial

biopsies in the setting of cardiac rejection demonstrate the presence of mononuclear cells, B and T lymphocytes as well as the presence of increased levels of a number of proinflammatory cytokines that may lead to cardiac failure. Interestingly, at the histological level a biopsy from a patient with acute myocarditis and that of a heart transplant recipient with rejection may be indistinguishable. Furthermore, both conditions can produce reversible myocardial dysfunction. Thus, the clinical settings of acute myocarditis and acute cardiac rejection not only demonstrate that active inflammation is detrimental to cardiac function, but also that if successfully treated, may be potentially reversible (Fig. 3).

1.4.2.2 Immune activation secondary to cardiac injury

Immune activation secondary to cardiac injury occurs when new antigenic epitopes are present in the myocardium and are capable of triggering an immune response. For example, following a myocardial infarction there are a number of inflammatory cells that populate the area of infarction. There are also a number of autoantibodies that are present in the serum that are directed against cardiac specific proteins. For instance, antibodies against myosin are found in patients with heart failure and in experimental animals immunization with myosin leads to the development of myocarditis (Neu, Rose et al. 1987; Kohno, Takagaki et al. 2001) indicating the potential pathogenetic role of these antibodies (Caforio, Goldman et al. 2001; Warraich, Noutsias et al. 2002). Also, antibodies against the [beta]-receptor are present in patients with advanced nonischemic cardiomyopathy and it has been suggested that clinical improvements in patients that have high antibody titers are related to the disappearance of antibodies from the circulation (Clark, Cleman et al. 2001). In addition to the presence of autoantibodies, there is evidence of activation of the complement system (Clark, Cleman et al. 2001), increased expression of class II major histocompatibility molecules (Levine, Kalman et al. 1990; Torre-Amione, Kapadia et al. 1995) and increased expression of intracardiac cytokines (Bryant, Becker et al. 1998) in patients with advanced heart failure. Thus, regardless of the origin of initial insult, myocardial injury can induce immune activation (Fig. 3).

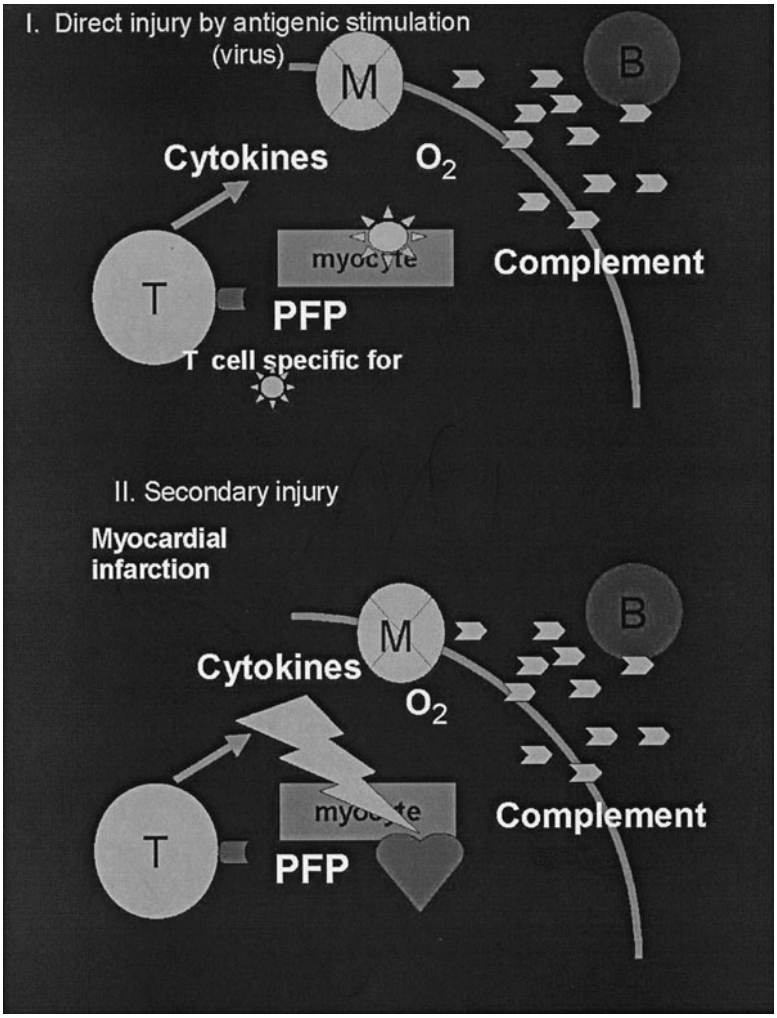


Figure 3. **Pathways of immune activation in chronic heart failure.** Cardiac immune injury is the result of activation of the immune system against virally infected cells, as occurs in patients with myocarditis (direct antigenic stimulation), or against the new expression of self-antigens that, after myocardial injury (eg, after a myocardial infarction), become antigenic (secondary injury). The mechanisms that follow the initial immune response are similar and involve the development of T cell-specific responses as well as antibody responses and complement activation. B = B cells; M = macrophage/monocyte; PFP = pore-forming (Torre-Amione 2005).

1.4.2.3 Cytokine release by cardiac cells

Recent experimental evidence suggests that myocardial cells are capable of producing a variety of cytokines following hemodynamic load. In a hypertensive murine model induced by infusion of Ang II, L-NAME and increased salt intake mice developed cardiac dysfunction in association with a wide array of activation of proinflammatory cytokines and a decrease in anti-inflammatory cytokines with

little or no inflammatory infiltrates, indicating that the hemodynamic load alone is capable of producing cardiac inflammation (Celis, Torre-Martinez et al. 2008).

1.4.3 Cytokines for the immune activation in CHF

CHF is characterized by increases in circulating proinflammatory cytokines (TNF-α, IL-6, IL-1β, and IL-2) and their soluble receptors or receptor antagonists that become more pronounced as myocardial function deteriorates (Huber-Lang, Younkin et al. 2002; Riedemann, Guo et al. 2002; Piagnerelli, Boudjeltia et al. 2003). Cytokines are small molecules that interlink, amplify and propagate the immune response, and are involved in recruiting cells to areas of inflammation, stimulating cell division, proliferation and differentiation (Torre-Amione 2005). Cytokines, when expressed at sufficiently high concentrations, such as those that are observed in heart failure, are sufficient to mimic some aspects of the heart failure phenotype, including progressive left ventricular dysfunction, pulmonary edema, left ventricular remodeling, fetal gene expression, and cardiomyopathy (Thaik, Calderone et al. 1995; Seta, Shan et al. 1996; Kubota, McTiernan et al. 1997; Bozkurt, Kribbs et al. 1998). Thus, heart failure may progress, at least in part, as a result of the effects exerted by endogenous cytokines on the heart and peripheral circulation.

These cytokines known to undergo up-regulation in patients with CHF have been implicated in the pathophysiology of this disease. Cytokines can affect myocardial function via the effects on both the myocyte contractility and the extracellular matrix. In addition to their effect on myocardial remodeling, cytokines have been shown to have direct and indirect effects on myocardial function (Prabhu 2004).

Proinflammatory cytokines affect cardiac mechanical function in a temporally dependent, biphasic manner. The early phase is characterized by the rapid activation of cellular signaling mechanisms that are likely interrelated, involving sphingolipid, phospholipid, and constitutive nitric oxide synthetase (NOS)-derived NO-dependent pathways. This response may be either cardiostimulatory or cardiodepressant, depending on the prevailing cellular and

biological milieu, specifically with regard to the redox and metabolic state, the magnitude of extracardiac adaptive and reflex responses, and the synergistic and/or counter-regulatory effects of several cytokines acting in concert. The early response is subsequently followed by a more prolonged, delayed phase that uniformly demonstrates depression of both basal and stimulated contractility (Prabhu 2004).

1.5 TNF-α and heart failure

Cytokines are essential to propagate and magnify the immune response. Cytokines are involved in recruiting cells to the area of inflammation to stimulate cell division, proliferation, and differentiation. Accordingly, cytokines - particularly those that promote inflammation - are key elements of immune activation. One of the best characterized inflammatory molecule in CHF is TNF-α.

1.5.1 TNF-α

TNF, an endotoxin-induced serum factor that caused necrosis of tumors, was first identified in 1975 (Carswell, Old et al. 1975). A decade later, investigators isolated a protein from endotoxin-treated cells that was named cachectin because of its presumed role in the molecular basis of cachexia (Beutler, Mahoney et al. 1985; Evans, Argiles et al. 1989). The subsequent cloning of the genes encoding cachectin and TNF-α confirmed that these two molecules were identical (Pennica, Hayflick et al. 1985; Caput, Beutler et al. 1986). TNF-α is a trimeric 17-kDa polypeptide produced mainly by monocytes and macrophages. In response to a wide variety of infectious or inflammatory stimuli (e.g., lipopolysaccharide, viruses, fungal or parasitic antigens, IL-1, TNF-α), both transcription and translation of TNF precursor is increased, and large amounts of mature protein are rapidly released into the circulation. TNF regulates the expression of a variety of proteins including IL-1, IL-6, platelet derived growth factor, transforming growth factor-beta, as well as a group of eicosanoids and hormones including platelet-activating factor and adrenaline (Tracey, Vlassara et al. 1989). In addition, TNF-α contains a 33 nucleotide 3′-untranslated sequence

that shortens mRNA half-life and limits the production of large quantities of this peptide (Caput, Beutler et al. 1986).

Normal myocardium does not contain TNF-α but expresses both of its receptors - TNF receptor type 1 and 2. However, in failing myocardium, there is increased expression of TNF-α, and the receptors for TNF-α are downregulated (Torre-Amione, Kapadia et al. 1995). Once released from the cell, TNF-α interacts with one of two TNF-α receptors that are expressed on many cell types (Torre-Amione, Bozkurt et al. 1999). The two receptors are a high-affinity receptor (soluble TNF receptor 1) and a low-affinity receptor (TNF receptor 2). The biologic activity of TNF-α can be attenuated by soluble TNF receptors. The cellular effects of TNF-α are highly pleiotropic. At low concentrations, TNF-α affects the paracrine or autocrine regulation of leukocytes and endothelial cells and thus serves as an important regulator of the inflammatory response. At higher concentrations, TNF-α production exceeds the number of TNF-α receptors located on the cell surface with excess TNF-α being released into the circulation. Once released, TNF-α exerts endocrine or exocrine effects, including initiation of metabolic wasting, microvascular coagulation, hypotension, and fever (Dinarello, Cannon et al. 1986; Feldman, Combes et al. 2000).

1.5.2 TNF-α participates in the development of CHF

The first recognition that TNF-α might participate in the development of CHF was reported in 1990 when circulating levels of TNF-α were demonstrated to be elevated in patients with end stage heart failure (Levine, Kalman et al. 1990). Subsequent study demonstrated a direct relationship between TNF-α levels and functional heart failure classification (Torre-Amione, Kapadia et al. 1996). Furthermore, an association between circulating levels of TNF-α and the prognosis in the patients with CHF were presented in the Vesnarinone trial, in which there is a significant overall difference in survival as a function of increasing TNF levels, with the worst survival in CHF patients with TNF levels greater than the 75th percentile (Kapadia, Torre-Amione et al. 1994; Dibbs, Thornby et al. 1999; Deswal, Petersen et al. 2001). In addition, direct relationships were identified between circulating levels of TNF-α, neurohumoral

activation and the degree of anemia; however, there was no relationship between cytokine concentrations and the degree of cachexia (Ferrari, Bachetti et al. 1995; MacGowan, Mann et al. 1997). TNF-α concentrations were also elevated in patients with heart failure due to myocarditis (Matsumori, Yamada et al. 1994). That the observed increases were of physiologic significance was demonstrated by studies in which injections of endotoxin into humans resulted in TNF-α elevations, depressed left ventricular function and decreases in mean arterial pressure (Suffredini, Fromm et al. 1989). Myocardial depression in septic shock is a well-defined entity. Myocardial dysfunction does not seem to be caused by myocardial hypoperfusion (Kumar, Haery et al. 2000) but rather by circulating depressant factors, cytokine TNF-α (Parrillo, Burch et al. 1985; Kumar, Wood et al. 2003).

TNF-α plays a crucial role in ischemia/reperfusion injury as a part of postresuscitation myocardial dysfunction. It has been reported that TNF-α increased threefold within 15 minutes of restoration of circulation and remained elevated throughout the observation period (Niemann, Garner et al. 2004). A significant negative correlation was observed between TNF-α and left ventricular systolic dP/dt. TNF-α concentrations increased dramatically shortly after resuscitation from ventricular fibrillation and remained elevated during the early postresuscitation period. A significant correlation was observed between TNF-α concentrations and left ventricular dP/dt, suggesting an association between TNF-α and postresuscitation myocardial contractility.

1.5.3 Mechanisms of upregulated synthesis of TNF-α in heart failure

1.5.3.1 Myocardial production

Experimental evidence of TNF-α synthesis in feline myocardium was initially observed in 1995 when the degree of distension of the left ventricular cavity was directly proportional to the local TNF-α production (Torre-Amione, Kapadia et al. 1995; Torre-Amione, Kapadia et al. 1996). Shortly afterwards, the presence of mRNA and cytokine receptors were firstly observed in human myocytes isolated

from necropsy hearts (Ferrari, Bachetti et al. 1995). Based on these observations, Torre-Amione et al considered a mechanism of myocardial production of TNF-α to justify the elevated levels of cytokines in heart failure, where the diastolic wall distention associated with increased filling pressures would lead to a local overexpression of TNF-α, and cytokines would spillover to the circulation, thus contributing to the immune activation and systemic inflammatory status (Torre-Amione, Kapadia et al. 1995; Torre-Amione, Kapadia et al. 1996) (Fig. 4).

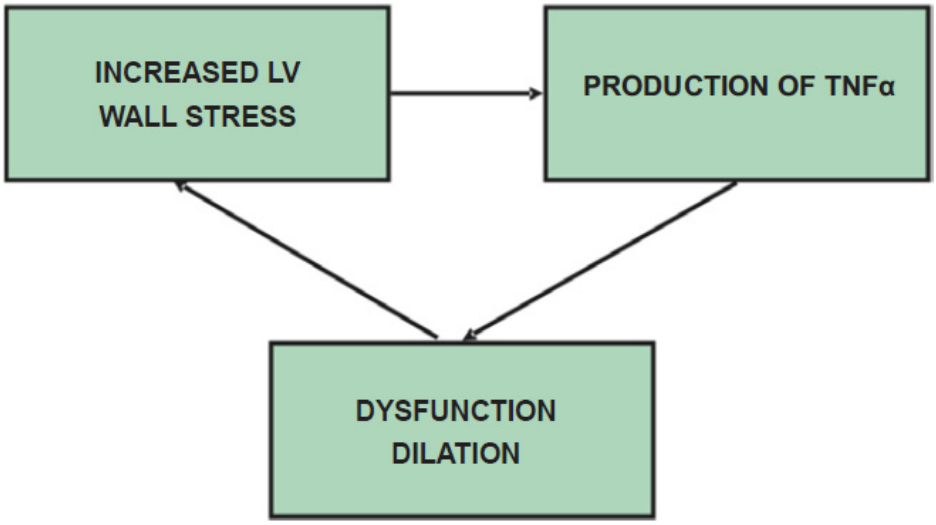


Figure 4. **Hypothesis of myocardial TNF-α production in heart failure** (Candia, Villacorta et al. 2007).

1.5.3.2 Extramyocardial production

Hasper et al hypothesized that the inefficient vasodilator response and the reduced aerobic enzyme activity characteristic of the multiorgan involvement of heart failure would be sufficient stimuli to cause a systemic TNF-α overexpression, notably in skeletal muscles (Hasper, Hummel et al. 1998). Tissue hypoxia and free radical generation are potent stimuli for the synthesis of TNF-α in immunocompetent cells of the whole body (Hasper, Hummel et al. 1998; Torre-Amione 2005); with the progression of the disease, elevated levels of TNF-α would worsen endothelial dysfunction, tissue hypoxia, and skeletal

muscle apoptosis even more, and this would serve as a stimulus for the systemic synthesis of TNF-α and oxidative stress, thus creating a vicious cycle of perpetuation of the disease and promotion of cachexia that is close to the model of multisystemic progression of heart failure (Tsutamoto, Hisanaga et al. 1998; Anker and Sharma 2002). These relations are shown in Figure 5.

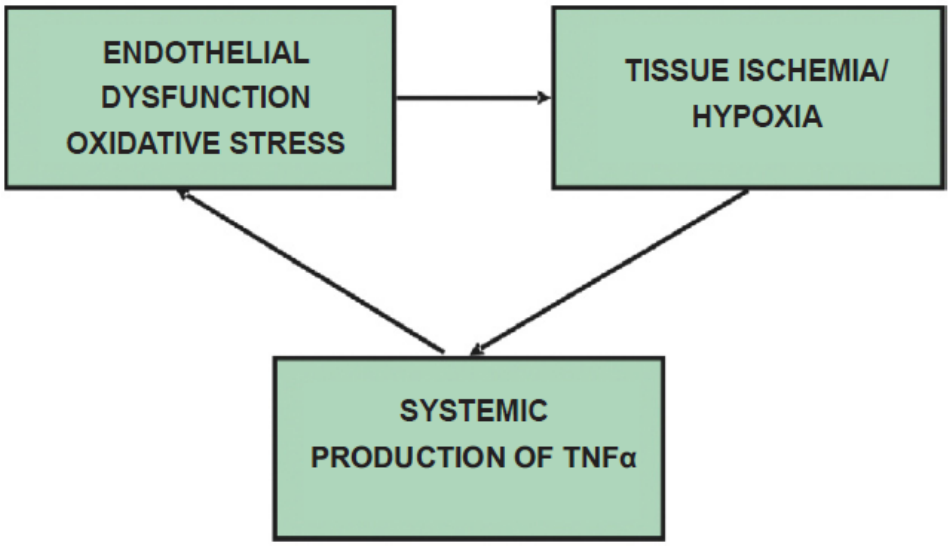


Figure 5. **Hypothesis of extramyocardial TNF-α production in heart failure** (Candia, Villacorta et al. 2007).

1.5.3.3 Intestinal production: the endotoxin-cytokine hypothesis

When Anker et al. observed a concomitant TNF-α and soluble CD14 receptor (sCD14) elevation in the peripheral blood of patients with advanced heart failure, they conceived a model of cytokine activation in heart failure that would necessarily involve the production of endotoxins derived from intestinal bacteria (Anker, Egerer et al. 1997). They analyzed the peripheral levels of these substances in 47 patients with advanced heart failure (29 of ischemic etiology) and in 17 healthy controls without structural heart disease or acute/chronic inflammatory conditions. The levels of sCD14 were increased in patients with heart failure, especially in cachectic ones, and a strong correlation between the levels of sCD14 and of TNF-α, soluble tumour necrosis factor receptor (sTNF-R) 1 and sTNF-R2 was observed – thus suggesting that endotoxins (ETX) were

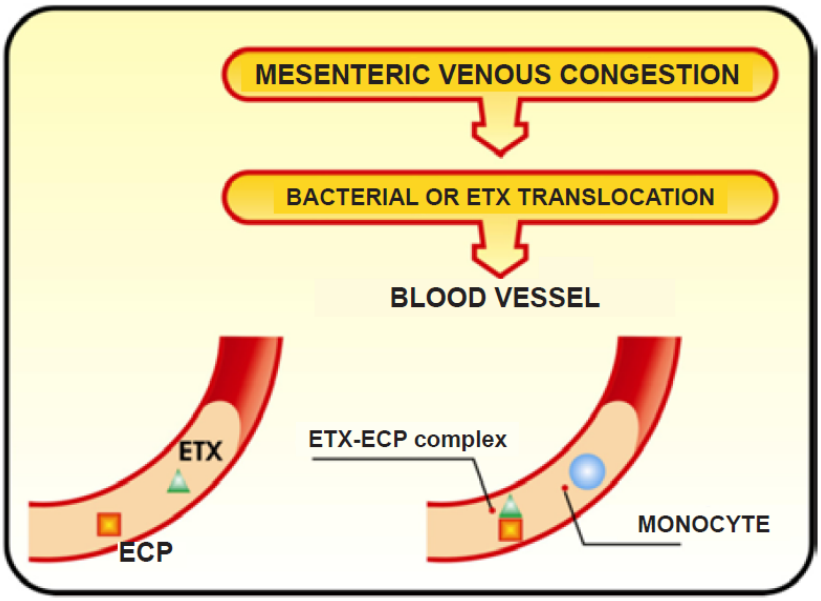
involved in the immuneinflammatory activation in heart failure (Anker, Egerer et al. 1997).

Thus, Anker et al. hypothesized that, in patients with heart failure, the interaction between CD14 receptors of immunocompetent cells and ETX released by gram-negative bacteria (GNB), from the gastrointestinal tract, would result in the activation of monocytes (Anker, Egerer et al. 1997). This interaction is actually documented as the most potent endogenous reaction capable of releasing TNF-α (Anker, Egerer et al. 1997; Niebauer, Volk et al. 1999; Torre-Amione 2005). With the purpose of corroborating the initial hypothesis, these investigators later demonstrated that patients with heart failure and peripheral edema had higher levels of sCD14, TNF-α and ETX than those without edema, and the latter presented higher levels than healthy controls without heart failure (Niebauer, Volk et al. 1999). Moreover, after a mean 40-day treatment with diuretics, a significant decrease in ETX levels and a tendency of decrease in TNF-α levels were observed (Niebauer, Volk et al. 1999).

Anker et al. suggested that their results supported the hypothesis that in severe CHF the congestion of the intestinal wall would allow a translocation and/or ETX release in the bloodstream (Anker, Egerer et al. 1997). After translocation there would be immune activation via binding of circulating ETX to CD14 receptors, with release of sCD14 into the bloodstream (Anker, Egerer et al. 1997), which can be detected in plasma (Brunkhorst, Clark et al. 1999; Niebauer, Volk et al. 1999; Krack, Sharma et al. 2005). This mechanism of immume activation is called the “endotoxin-cytokine hypothesis” (Krack, Sharma et al. 2005). The cellular and subcellular mechanisms of this hypothesis are shown in Figures 6A and 6B.

Recently, a pilot study has shown that a therapeutic potential of selective intestinal decontamination (SID) on the inflammatory activation in advanced heart failure. SID regimen with nonabsorbable antibiotics (polymyxin B and tobramycin for eight weeks) was able to eradicate intestinal GNB and significantly reduce blood and fecal levels of ETX, as well as serum levels of IL1, IL6 and TNF-α in patients with NYHA functional class III and IV (Conraads, Jorens et al. 2004).

A.



B.

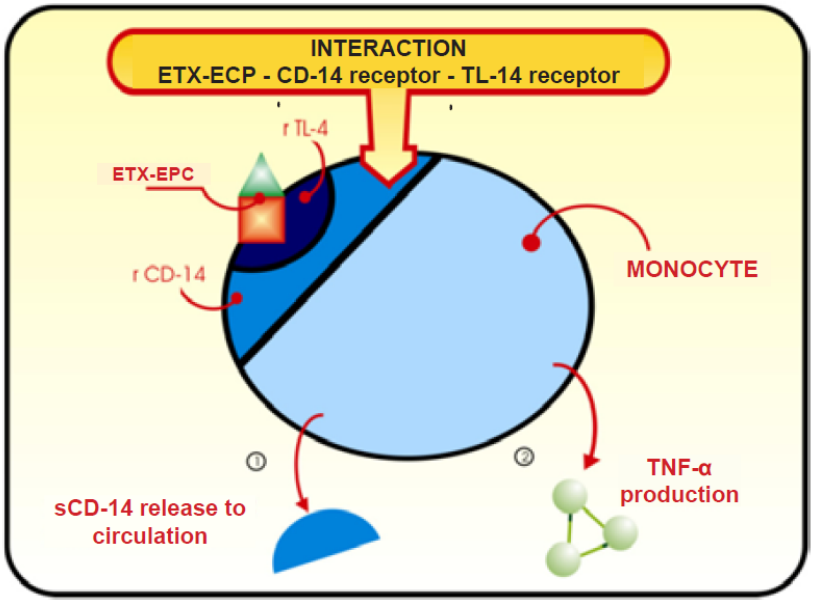


Figure 6. **Cellular (A) and subcellular (B) mechanisms related to the immuneinflammatory activation by serum ETX in heart failure**. ETX - endotoxin; ECP - ETX carrier protein; rTL-4 - tool-like receptor 4; rCD14 - CD14 receptor; sCD14 – soluble portion of CD14 receptor (Candia, Villacorta et al. 2007).

1.5.4 Biologic effects of TNF-α on the myocardium

Beginning with the observation that TNF-α could inhibit contractility of isolated hamster papillary muscles in a concentration-dependent and reversible manner (Finkel, Oddis et al. 1992), a series of in vivo and in vitro studies examining the effects of TNF-α on the myocardium were performed. First, it was demonstrated that the negative inotropic effects of TNF-α are virtually immediate (Eichenholz, Eichacker et al. 1992; Yokoyama, Vaca et al. 1993) and appear to be completely reversible upon removal of the cytokine. However, TNF-α does not only have negative inotropic effects, but it can mimic cellular and biochemical abnormalities that characterize the failing human heart. In addition, TNF-α effectively uncouples the beta-adrenergic receptors from adenylyl cyclase via an effect on the G inhibitory protein (Gulick, Chung et al. 1989; Chung, Gulick et al. 1990). Furthermore, TNF-α activates metalloproteinases and inhibits the expression of inhibitors of metalloproteinases in vivo - effects that would be expected to activate extracellular matrix remodeling (Li, McTiernan et al. 1999). Although initial studies suggested that the negative inotropic properties of TNF-α were attributable to the ability of cytokines to induce NOS, subsequent investigations demonstrated that the induction of inducible NOS is not sufficient to induce contractile dysfunction in cardiac myocytes and that alterations in calcium homeostasis play an important role (Yokoyama, Vaca et al. 1993). TNF-α also provokes a hypertrophic growth response in cardiac myocytes, which may be an adaptive response to hemodynamic or environmental stress (Yokoyama, Nakano et al. 1997). Furthermore, a recent study suggested that the immediate negative inotropic effects of TNF-α may be mediated by sphingosine (Oral, Dorn et al. 1997). Finally, chronic infusion of TNF-α produces a reversible dilated cardiomyopathy in a rat model - without evidence of inflammatory infiltrate or myocyte necrosis (Bozkurt, Kribbs et al. 1998).

1.6 Monocytes in the immune activation in heart failure

Monocytes play an important role in immune defence, inflammation, and tissue remodelling. The function of monocytes involves phagocytosis, antigen

processing and presentation, and cytokine production (Swirski, Pittet et al. 2006). Monocytes consist of a heterogeneous multifunctional cellular population. They represent both the main cellular source and one of the main cellular targets of pre-inflammatory cytokines (Table 1) (Dutka, Elborn et al. 1993; Seta, Shan et al. 1996; Bozkurt, Kribbs et al. 1998; Nakano, Knowlton et al. 1998; Deswal, Petersen et al. 2001; Lecour, Smith et al. 2002; Chung, Packer et al. 2003; Maack, Kartes et al. 2003; Mann, McMurray et al. 2004; Sun, Dawood et al. 2004; Armstrong, Morrow et al. 2006; Kjekshus, Apetrei et al. 2007). However, their role in heart failure is not restricted to cytokine signaling. There is good evidence implicating monocytes in various cardiovascular disorders associated with heart failure.

Under certain stimuli, circulating monocytes differentiate into macrophages. Macrophages are mobile, phagocytic cells specialized in removing unwanted cellular and extracellular debris, invading microorganisms, and other foreign material (Swirski, Weissleder et al. 2009).

Monocytes are involved in atherosclerosis, the pathophysiological process underlying coronary artery disease and subsequently of ischaemic cardiomyopathy (Ross 1999). Their initially protective inflammatory response starts to damage the arterial wall, and dysfunctional endothelial cells (ECs) and macrophages release cytokines, chemokines, and growth factors, which promote the migration of smooth muscle cells into the intima of the arterial wall forming the intermediate or fibro-fatty lesion. At this stage, the lesion can contain multiple layers of smooth muscle cells, connective tissue, macrophages, and T cells (Ross 1999; Apostolakis, Vogiatzi et al. 2008). Remodelling of the vessel wall occurs, resulting in advanced lesion formation.

Table 1 Cytokines/chemokines produced by monocyte/macrophages in response to inflammation

Cytokine	Major action in relation to vascular inflammation
TNF-α (Dutka, Elborn et al. 1993; Seta, Shan et al. 1996; Torre-Amione, Kapadia et al. 1996; Bozkurt, Kribbs et al. 1998; Nakano, Knowlton et al. 1998; Deswal, Petersen et al. 2001; Lecour, Smith et al. 2002; Chung, Packer et al. 2003; Maack, Kartes et al. 2003; Mann, McMurray et al. 2004; Sun, Dawood et al. 2004; Armstrong, Morrow et al. 2006; Kjekshus, Apetrei et al. 2007)	Induces inflammation Induces apoptotic cell death Potent chemoattractant for neutrophils
IL-1α/β (Apostolakis, Vogiatzi et al. 2008)	Induces chemokine/cytokine expression Promotes the expression of adhesion molecules on ECs
IL-6 (Seta, Shan et al. 1996; Mann 2002)	Induces chemokine/cytokine production Major mediator of the acute phase response
IL-8 (Apostolakis, Vogiatzi et al. 2008)	Neutrophil activation and chemotaxis SMCs proliferation and migration
IL-10 (Seta, Shan et al. 1996; Mann 2002)	Anti-inflammatory cytokine Down-regulates the expression of Th1 cytokines Inhibits NFκB activity
IL-12 (Swirski, Weissleder et al. 2009)	Induces cell-mediated immunity T cell stimulating factor Induces the production of TNF-α and INF-γ
IL-18 (Seta, Shan et al. 1996; Mann 2002; Apostolakis, Vogiatzi et al. 2008)	Induces cell-mediated immunity Induces INF-γ production
MIP 1α/β (Seta, Shan et al. 1996; Mann 2002; Apostolakis, Vogiatzi et al. 2008)	Activate and chemotaxis of human granulocytes Induces the synthesis and release of pro-inflammatory cytokines
MCP 1 (Seta, Shan et al. 1996; Mann 2002; Apostolakis, Vogiatzi et al. 2008)	MCs chemotaxis and activation Induces SMCs accumulation and migration

TNF, tumour necrosis factor-α; IL, interleukin; EC, endothelial cells; INF, interferon; MIP, monocyte inflammatory protein; MCP, monocyte chemoattractant protein; MC, monocytes; SMC, smooth muscle cells.

Ischaemia-induced myocardial damage and subsequently myocardial remodelling and heart failure have also been associated with intense inflammatory activity and monocyte infiltration in the damaged tissue. In the early 1980s the mainstream theory indicated a rather catastrophic role of monocyte accumulation in the ischaemic myocardium, whereby hypoxia induces

necrosis in the cardiac myocytes, which subsequently stimulates the complement cascade and initiates an inflammatory response. Once activated, monocytes/macrophages produce several cytokines, chemokines, and growth factors, including IL-1α and -β, IL-6, TNF-α, and macrophage inflammatory proteins 1 α/β (Roberts, Maclean et al. 1985; Swirski, Weissleder et al. 2009). The theory was further supported by studies in animal models showing that the infarct size was reduced if blood was depleted of white cells or if lipoxygenase metabolism of arachidonic acid was inhibited (Ono, Matsumori et al. 1999). More recent studies have suggested a deteriorating effect of monocyte infiltration in myocardial tissue. For example, a strong association between peripheral monocytosis, left ventricular dysfunction, and left ventricular aneurysm formation after myocardial infarction is observed (Maekawa, Anzai et al. 2002). Consequently, inhibition of monocytes activation is a tempting therapeutic target in the prevention of ischaemic heart failure.

2 Aim

Plasma CT-1 levels increase with the severity of CHF. However, the role of CT-1 in the pathophysiology of CHF remains unclear. CT-1 is a strong acute-phase mediator for hepatocytes in vitro. Its activity is similar to LIF on hepatocytes, H35 cells, and HepG2 cells that induces a dose-dependent production of several acute phase proteins (haptoglobin, fibrinogen, alpha1-acid glycoprotein, alpha2-macroglobulin), suggesting that CT-1 could play an important role in the regulation of the inflammatory process (Robledo, Guillet et al. 1997). As immuneinflammatory activation is associated with the progression of CHF, it is possible that CT-1 might play a role in modulating the immune response and cytokine activation in CHF, which in turn might contribute to the deterioration and progression of CHF. In addition, peripheral blood mononuclear cells (PBMC) are a critical component in the immune system and widely used in the research of inflammatory responses. It has been demonstrated that PBMC were able to produce TNF-α in CHF (Vonhof, Brost et al. 1998; Zhao and Xu 1999). But so far the mechanism responsible for TNF-α production in these cells under these circumstances is not fully understood.

Given the above considerations, I hypothesized that CT-1, which is elevated in CHF, would activate PBMC and promote the expression of TNF-α in them. To test the above hypothesis, I propose to isolate and purify PBMC from human whole blood, and culture the cells in vitro. The first goal would be to test whether gp130/LIFR-β is expressed in PBMC. If gp130/LIFR-β is demonstrated to be expressed in them my next goal would be to elucidate whether CT-1 can activate PBMC by both signaling pathway and cytokines production. Further if PBMC demonstrated to be activated by CT-1 my future goal would be to test whether CT-1 can promote the expression of TNF-α in PBMC and identify the subsets of PBMC which is responsible for the production of TNF-α. Furthermore, the underlying signaling pathways responsible for CT-1 induced TNF-α production in PBMC will be tested.

The present work will reveal a new mechanism of immune response and cytokine activation in heart failure and emphasize the role of CT-1 in the

pathophysiology of CHF. It will illustrate that the heart is not only just a target of inflammation in heart failure, but also plays a role in the activation of inflammatory response by secreting CT-1 into the peripheral circulation, at least in part as a cause for initiating or promoting the inflammatory response in heart failure, and therefore indicate that modulating CT-1 may be an interesting pharmacological target in the treatment of CHF.

3 Material and Methods

3.1 Reagents

Chemicals. All general chemicals were purchased from *Carl Roth GmbH & Co.*, Karlsruhe, *Merck*, Darmstadt or *Sigma Chemie GmbH*, Deisenhofen and were of the highest quality.

Antibody. The blocking antibody against CT-1 and gp130 were purchased from R&D Systems (Wiesbaden, Germany). Monoclonal antibodies against inhibitor of kappa B (IκB), LIFR-β, STAT-1, STAT-3, ERK, P38 MAPK, JNK and the phosphorylated STAT-1, STAT-3, ERK, P38 MAPK, JNK were from BD-Pharmingen (Heidelberg, Germany), horseradish peroxidase (HRP) -conjugated goat anit-mouse and goat anti-rabbit IgG secondary antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fluorescein Isothiocyanate (FITC) -conjugated monoclonal antibody against the surface antigens cluster of differentiation (CD) 3 was purchased from Coulter-Immunotech (Krefeld, Germany), CD4 from Caltag (Hamburg, Germany), CD8 and CD14 from BD-Pharmingen (Heidelberg, Germany). Phycoerythrin (PE) -conjugated monoclonal antibodies against IL-2, IL-4, IL-5, IL-10, interferon (IFN) -γ and TNF-α were purchased from BD-Pharmingen (Heidelberg, Germany).

Other Reagents. Recombinant human CT-1 was purchased from R&D Systems (Wiesbaden, Germany) and dissolved according to the manufacturer´s instruction. Human vein endothelial cells were purchased from PromoCell (Heidelberg, Germany). Actinomycin D, brefeldin A, parthenolide, and PD98059 were purchased from Sigma chemicals (Deisenhofen, Germany), SB203580 from Upstate (Dundee, UK). PBMC separation medium Ficoll-Paque Plus from Amersham Bioscience (Uppsala, Sweden). RPMI 1640 from Cell Concepts (Umkirch, Germany). Fetal calf serum from PromoCell (Umkirch, Germany) or Cell Concepts (Heidelberg, Germany). Penicillin/streptomycin from Biochrom AG (Berlin, Germany). EpiQuik^{TM} Nuclear Extraction KIT I were purchased from Epigentek (New York, USA). High Pure RNA Isolation Kit from Qiagen (Hilden, Germany). Reverse transcription kit from Promega (Madison, USA). Fast Start DNA Master SYBR Green I kit from Roche Diagnostics (Mannheim, Germany).

TNF-α ELISA QuantiGlo from R&D Systems (Wiesbaden, Germany). Electrophoretic-Mobility Shift Assay (EMSA) Kits and probes were purchased from Panomics (Redwood City, USA).

3.2 Cell culture

3.2.1 Isolation of human PBMC from whole blood

3.2.1.1 Blood collection

The PBMC were obtained exclusively from the blood of healthy volunteers. Venous blood (drawn in the morning from 9.00 to 11.00 h) were collected in EDTA tubes (1.6 mg EDTA / ml blood) (SARSTEDT AG & Co, Nümbrecht, Germany) from a peripheral forearm vein.

All steps of isolation, incubation and stimulation of the cells were performed under sterile conditions in a laminar flow hood (HERA Safesteril Bank, Heraeus Instruments, Gera).

3.2.1.2 Ficoll-Paque density centrifugation

Separation medium (Ficoll-Paque Plus, Amersham Bioscience, Uppsala, Sweden) and sterile PBS were warmed up in room temperature. Six Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany) were filled with 15 ml separation medium. After centrifugation at 1000 × g at room temperature for 30 sec, 50ml of 1: 2 diluted anticoagulated blood sample was poured into the Leucosep tube (25ml per tube). After centrifugation at 1000 × g at room temperature for 10 min, an enriched cell fraction was located at the interface between plasma and separation medium as a cloudy whitish layer. The enriched cell fraction was harvested in another 50 ml tube. After centrifugation at 1500 rpm at room temperature for 10 min, the supernatant was removed, then 10 ml of 0.83% NH_4Cl was added to lyse red blood cells. Centrifugated at 1500 rpm at room temperature for 10 min, the supernatant was removed, and the cell pellet was suspended in 10 ml PBS and fresh PBS was added to an final volume of 50

ml. Then the cell suspension was centrifugated at 1500 rpm at room temperature for 10 min.

3.2.1.3 Incubation and stimulation of cells

The cells were washed three times with PBS, resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% penicillin, streptomycin, and cultivated in plastic dishes at 37°C in a humified 5% CO_2 atmosphere for 24 hrs. Afterwards, medium was replaced by fresh medium. After 24 hours, over 90% of PBMC were alive tested by trypan blue exclusion. Stimulation and pharmacological studies were done afterwards.

Primary cultures from human vein endothelial cells were done according to the manufacturer`s manual in endothelial growth medium with 2% FCS. Cells were grown to confluence in collagen I coated tissue culture plastic (Becton Dickinson, Franklin Lakes, USA). Cells were used in the second to fifth cell passages.

All stimulants, inhibitors and media were without endotoxin levels according to the manufacturers' instructions.

Pharmacological agents, dissolved in fresh medium, were added to the cells for defined time intervals and concentrations. As a control, fresh medium was added to the cells.

Approval of this study was given by the Ethics Committee of the Friedrich-Schiller-University, Jena, and subjects gave their written informed consent according to University guidelines.

3.3 Preparation of protein probes and nuclear protein extracts

3.3.1 Protein probes

Depending of the application, medium was removed and 2× laemmli buffer was added (160 mM Tris.HCl pH 6.8, 4% SDS, 16% glycerol, and 0.005% bromophenol blue). Afterwards the cells were scraped and transferred to a 1.5 ml reaction tube and the genomic DNA was sonified (Branson cell disruptor B15,

output 6) to reduce viscosity. Before loading the samples, 100 mM dithiothreitol (DTT) was added and proteins was denatured by heating for 3 min at 95°C.

3.3.2 Nuclear protein extraction

Nuclear extracts were achieved by the EpiQuik^{TM} Nuclear Extraction KIT I according to the manufacturer's manual. Briefly, cells were grown to 70-80% confluence on a culture plate (about 2-5 × 10^6 cells for a 100 mm plate). The growth medium was removed, cells were washed twice with PBS and then PBS was removed. The cells were added with 1 ml of fresh PBS per 20 cm^2 area, and scraped into a 15 ml conical tube. After centrifugation at 1000 rpm for 5 min, the supernatant was discarded, and the cell pellet was re-suspended in 100 µl of diluted NE1 (10% NE1, 90% H_2O, 0.1% DTT and 0.1% PIC) per 10^6 cells and transferred to a micro centrifuge vial. After incubation on ice for 10 min, the vial was vortexed vigorously for 10 sec and centrifuged for 1 min at 12,000 rpm. The cytoplasmic extract was carefully removed from the nuclear pellet. Twenty µl of NE2 (containing 0.1% DTT and 0.1% PIC) was added to the nuclear pellet. The extract was incubated on ice for 15 min with vortex (5 sec) every 3 min. The extract was sonicated 3 times, each 10 sec, to increase nuclear protein extraction. After centrifugation at 14,000 rpm at 4°C for 10 min, the supernatant was transferred into a new microcentrifuge vial. Protein concentration of the nuclear extract was measured, and then the nuclear extract was used immediately or aliquoted and freezed at -80°C until further use.

3.4 Western blot analysis

3.4.1 Separation of proteins by polyacrylamide gel electrophoresis (PAGE)

Unless otherwise indicated for most applications a polyacrylamide separating gel of 10-12% was made and a 5% stacking gel. Reagents for the stacking gel were 8.3 ml of acrylamide/bis-acrylamide (30:0.8) 6.25 ml of 1.5 M Tris-HCl pH 8.8, 0.124 ml of 20% sodium dodecyl sulfate (SDS), an 10.05 ml of H_2O (for a

12% separating gel, the amount of acrylamide/bis-acrylamide added was 10 ml and the H_2O reduced accordingly to give the same end volume). To the mixture was added 250 µl of 10% ammonium persulphate (APS) and the reaction initiated with 20 µl of tetramethylethylenediamine (TEMED). The gel mix was poured between two glass plates with spacers between and allowed to polymerize. Upon completion, a stacking gel was poured on top. This was made up of 1.7 ml of acrylamide/bis-acrylamide, 2.5 ml of 0.5 M Tris-HCl pH 6.8, 50 ml of 20% SDS, 5.65 ml of H_2O, 100 µl of 10% APS and 7.5 µl of TEMED. The gel was then run in 1 laemmli-running buffer (25 mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1% SDS) until the desired distance had been reached.

3.4.2 Immunoblotting

After gel electrophoresis proteins were transferred to Immobilon membranes (Millipore Corporation). The blots were incubated in blocking buffer, 10% skimmed milk, 0.1%Tween in 10 mM Tris pH 7.6, 100 mM NaCl (TBS) for 1 hour at room temperature. Incubation with primary antibodies was in blocking buffer (for phopho specific antibodies 5% bovine serum albumin (BSA), 0.1% Tween in TBS) for 1 hour room temperature (for phosphospecific antibodies overnight 4°C). Washed in TBS 0.1% Tween 3 times, each 10 min. Incubation with peroxidase conjugated secondary antibodies was in blocking buffer for 1 hour room temperature. Washed in TBS 0.1% Tween 3 times, each 10 min. Blots were developed using enhanced chemiluminescence system (Amersham, Little Chalford, England) and visualized with autoradiography film (Fuji Photo Film Co. Ltd).

3.5 Isolation of total ribonucleic acid (RNA) from cultured cells

Total RNA from cultivated PBMC was extracted according to the protocol of High Pure RNA Isolation Kit. Briefly, the growth medium was removed and cells were resuspended with 200 µl PBS and then added 400 µl Lysis/-Binding Buffer (green cap) and vortexed for 15 sec. Sample was pipeted into the upper reservoir of the Filter Tube (max. 700 µl). After centrifugation of the tube

assembly at 8,000 × g for 15 sec, the Filter Tube was removed from the Collection Tube. The flowthrough liquid was discard, and the Filter Tube was again combined with the used Collection Tube. One hundred µl mixture per sample (90 µl deoxyribonuclease (DNase) Incubation Buffe and 10 µl DNase I) was pipeted on the glass filter fleece in the upper reservoir of the filter tube. After incubation for 15 min at 15 to 25°C, 500 µl Wash Buffer I was added to the upper reservoir of the Filter Tube assembly and centrifuged at 8,000 × g for 15 sec. The flowthrough was discarded and the Filter Tube was again combined with the used Collection Tube. Five hundred µl Wash Buffer II was added to the upper reservoir of the Filter Tube assembly and centrifuged at 8,000 × g for 15 sec. The flowthrough was discarded and the Filter Tube was again combined with the used Collection Tube. Two hundred µl Wash Buffer II was added to the upper reservoir of the Filter Tube assembly and centrifuged at maximum speed (approx. 13,000 × g) for 2 min to remove any residual Wash Buffer. The Collection Tube was discarded and then the Filter Tube was inserted into a clean, sterile 1.5 ml microcentrifuge tube. Fifty to one hundred µl Elution Buffer was added to the upper reservoir of the Filter Tube. The tube assembly was centrifuged at 8,000 × g for 1 min. Then the microcentrifuge tube contained the eluted RNA. The eluted RNA was used directly in reverse transcription polymerase chain reaction (RT-PCR) or stored at -80°C for later analysis.

3.6 Reverse transcription from RNA into complementary deoxyribonucleic acid (cDNA)

One µg of total RNA was reversely transcribed into cDNA in a volume of 20 µl with avian myeloma leukaemia virus (AMV) reverse transcriptase and oligo dT primers (Promega, Madison, USA) according to the manufacturer`s manual. Briefly, 1 µg of 1.2kb Kanamycin Positive Control RNA, poly(A)+ mRNA or total RNA was placed in a microcentrifuge tube, and incubated at 70°C for 10 min. After centrifugation briefly in a microcentrifuge,the tube was placed on ice. A 20 µl reaction was prepared by adding the following reagents: 4 µl of 25 mM $MgCl_2$, 2 µl of reverse transcription 10X Buffer, 2 µl of 10 mM dNTP mixture, 0.5 µl of recombinant RNasin® ribonuclease inhibitor, 15 U AMV reverse transcriptase

(High Conc.), 0.5 µg Oligo(dT)15 primer, and nuclease-free water to a final volume of 20 µl. The reaction was incubated at 42°C for 15 min and heated at 95°C for 5 min, and then incubated at 0-5°C for 5 min.

3.7 Real-time PCR

3.7.1 Principle of the assay

Generation of PCR products can be detected by measurement of the SYBR Green I fluorescence signal. SYBR Green I intercalates into the DNA double helix. In solution, the unbound SYBR Green I dye exhibits very little fluorescence; however, fluorescence (wave length, 530nm) is greatly enhanced upon DNA-binding. Therefore, during PCR, the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated.

As SYBR Green I dye is very stable (only 6% of the activity is lost during 30 amplification cycles) and the LightCycler® Instruments' optical filter set matches the wave lengths of excitation and emission, it is the reagent of choice when measuring total DNA.

3.7.2 Assay procedure

Real-time PCR measurement of TNF-α cDNA was performed with the Light Cycler Instrument using the Fast Start DNA Master SYBR Green I kit. For verification of the correct amplification product, PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. The specific primer for human TNF-α was purchased from R&D Systems. The amplification program for TNF-α consisted of 1 cycle of 94°C with a 4 min hold followed by 40 cycles of 95°C with a 45 sec hold, 59°C annealing temperature with a 45 sec hold and 72°C with a 45 sec hold. The specific primer pair for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was: sense primer 5´ GGG AAG GTG AAG GTC GG 3´, antisense primer 5´ TGG ACT CCA CGA CGT ACT CAG 3´. The amplification program for GAPDH consisted of 1 cycle of 95°C with a 30 sec hold

followed by 30 cycles of 95°C with a 5 sec hold, 59°C annealing temperature with a 10 sec hold and 72°C with a 20 sec hold. Each reaction (20 µl) contained 2 µl cDNA, 2.5 mM $MgCl_2$, 1 pmol of each primer and 2 µl of Fast Starter Mix (containing buffer, dNTPs, Sybr Green dye and Taq polymerase). Amplification was followed by melting curve analysis to verify the correctness of the amplicon. A negative control without cDNA was run with every PCR to assess the specificity of the reaction. Analysis of data was performed using Light Cycler software version 3.5. PCR efficiency was determined by analysing a dilution series of cDNA (external standard curve). The identity of the PCR product was confirmed by comparing its melting temperature (Tm) with the Tm of amplicons from standards or positive controls. GAPDH was analyzed in parallel to each PCR and the resulting GAPDH values were used as standards for presentation of TNF-α transcripts.

3.8 TNF-α enzyme-linked immunosorbent assay (ELISA)

Cultured PBMC were treated with different concentrations of CT-1 for various time periodes. TNF-α concentration in the culture supernatants was determined by ELISA according to the manufacturer´s instructions.

3.8.1 Principle of TNF-α ELISA assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of TNF-α bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

3.8.2 Assay procedure

- Sample collection and storage

  Cell culture supernates were collected and particulates were removed by centrifugation. Then the samples were assayed immediately or aliquoted and stored at -20°C.

- Reagent preparation

  - Wash Buffer - 100 ml of Wash Buffer Concentrate was diluted into deionized or distilled water to prepare 1000 ml of Wash Buffer;
  - Working Glo Reagent –1 part Glo Reagent A (4 ml) and 2 parts Glo Reagent B (8 ml) were mixed together 15 minutes to 4 hours before use. It was stored in a capped plastic container, protected from light. One hundred µl of the resultant mixture is required per well;
  - Calibrator Diluent RD5P (1×) - Twenty ml of Calibrator Diluent RD5P (5×) was diluted into 80 ml of deionized or distilled water to yield 100 ml of Calibrator Diluent RD5P (1×);
  - Standard - Reconstitute Standard with 0.5 ml of deionized or distilled water. This reconstitution produces a stock solution of 70,000 pg/ml. The standard was gently agitated for a minimum of 15 min prior to making dilutions. Nine hundred µl of Calibrator Diluent RD5P (1×) was pipeted into the 7000 pg/ml tube. Eight hundred µl of the appropriate Calibrator Diluent was pipeted into the remaining tubes. The stock solution was used to produce a 5-fold dilution series. The 7000 pg/ml standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/ml);

- Excess microplate strips were removed from the plate frame, returned to the foil pouch containing the desiccant pack, and resealed;

- One hundred µl of Assay Diluent RD1-27 was added to each well. Then, 100 µl of Standard, sample, or control was added per well. Covered with the adhesive strip provided. The assay was incubated for 3 hrs at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout was provided to record standards and samples

assayed. Each well was aspirated and washed by filling each well with wash buffer (400 µl) using a manifold dispense, repeating the process three times for a total of four washes. After the last wash, any remaining wash buffer was removed by decanting. The plate was inverted and blotted against clean paper towels. Two hundred µl of human TNF-α conjugate was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hrs at room temperature on the shaker. Repeated the aspiration/wash process as before four times. Then, 100 µl of Working Glo Reagent was added to each well and the plate was incubated for 5-20 min at room temperature on the benchtop by protecting from light. The relative light unit of each well was determined using a luminometer set with the following parameters; 1.0 min. lag time; 0.5 sec/well read time; summation mode; auto gain on.

3.9 Electrophoretic mobility shift assay (EMSA)

3.9.1 Principle of EMSA

Panomics Electrophoretic-Mobility Shift Assay (EMSA) Kits are useful tools for identifying proteins that interact with DNA. This rapid technique is based on the separation of free DNA from protein/DNA complexes due to the differences in their electrophoretic mobility in native (non-denaturing) polyacrylamide gels. When a protein binds specifically to a labeled double stranded deoxyribonucleic acid (dsDNA) sequence, it migrates slower than non-bound dsDNA in a polyacrylamide gel, thus resulting in discrete bands corresponding to the individual protein/DNA complex. A typical EMSA experiment is performed by incubating a biotin-labeled transcription factor (TF) Probe with treated and untreated nuclear extracts. The protein/DNA complexes are separated on a non-denaturing polyacrylamide gel. The gel is transferred to a nylon membrane and detected using strepatvidin-HRP and a chemiluminescent substrate. The shifted bands corresponding to the protein/DNA complexes are visualized relative to the unbound dsDNA. The bands are visualized after exposure to film or chemiluminescent-imaging system.

3.9.2 Assay procedure

- Preparing samples:

  A 10 µl reaction was prepared by adding the following reagents in a sterile 0.5 ml microcentrifuge tube: 5 µg of nuclear extract, 2 µl of 5x binding buffer, 1 µl of I M Poly d (I-C), and distilled water to a final volume of 7 µl for cold probe and 9µl for probe. After 5 min incubation at room temperature, 1µl of TF probe and 2 µl of cold TF probe was added to the reaction of cold probe, and 1µl of TF probe was to the reaction of cold probe. Both reactions have a final volume of 10 µl. Then the reactions were incubated at 15-20°C for 30 min.

- Gel preparation, loading and running:

  Reagents for the 6% polyacrylamide gel were 1 ml of 10xTBE, 4 ml of 30% Acrylamide/Bis, 625 µl of 80% Glycerol, 14.375 ml of dH_2O, 300 µl of 10% APS, and 20 µl of TEMED. The gel mix was poured between two glass plates with spacers between and allowed to polymerize. The gel was run in chilled 0.5× TBE for 10 min in ice bath at 120V before loading samples into gel. Samples were mixed with 1 µl of Loading Dye. Ten µl of sample pre lane was loaded. The gel was run in ice bath at 120 V until the dye reaches 2.5 cm from the bottom of the gel (about 50-55 min).

- Transfer:

  Standard “wet-transfer” electroblotting procedures were performed as below. Nylon membrane (7 × 9 cm) was presoaked in 0.5× TBE. The gel was sandwiched with the presoaked membrane and two sheets of presoaked Whatmann paper (8 × 10 cm) on each side. The sandwich was placed in an electroblotting device, filled tank with 0.5× TBE, and transferred for 30-45 min at 300 mA at room temperature.

- Immobilization of bound oligos:

  After transfer, the membrane was removed and baked for 1 hour at 80°C in a dry oven, and then transferred to a UV crosslinker oven for 3 min.

- Detection.

  The membrane was blocked by incubating at room temperature with 20 ml of 1× blocking buffer for 15 min with gentle shaking. One ml of the 1× blocking buffer was removed from the blot container to a new eppi tube, added 20 ul of Streptavidin-HRP conjugate, vortexed and transferred back into the 1× blocking buffer. After shake at room temperature for another 15 min, the diluted streptavidin-HPR solution was decanted. The membrane was washed 3 times with 20 ml of 1× wash buffer, each 8 min. Then it was added with 20 ml of 1× detection buffer and inculated at room temperature for 5 min. Each membrane was placed on a plastic sheet. Two ml of mixed substrate solution (containing 200 ul of Solution I, 200 ul of Solution II, and 1.6 ml of Solution III) was pipeted onto each membrane, and a second plastic sheet was overlaid on it. After incubation at room temperature for 5 min, the membranes was exposed by using either Hyperfilm ECL (2-10 min) or a chemiluminescent imaging system (Fujifilm LAS-3000 Imager, Fuji Photo Film Co., Ltd., Tokyo, Japan) (12-15 min).

3.10 Immunofluorescent flow cytometric analysis of cytokine production

3.10.1 Principle of flow cytometry

In flow cytometry, a beam of laser light of a single wavelength is directed onto a hydrodynamically-focused stream of liquid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors.

The analysis based on the specific interaction of antigen with (monoclonal) antibodies, which are conjugated to fluorescent dyes. During the analysis the suspended cells are hydrodynamical focused and pass, like a string of pearls, the focused laser beam. Each suspended cell passing through the beam scatters the ray, and fluorescent chemicals found in the cell or attached to the cell may be excited into emitting light at a longer wavelength than the light

source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual cell. FSC correlates with the cell volume and SSC depends on the inner complexity of the cell (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). (Fig. 7).

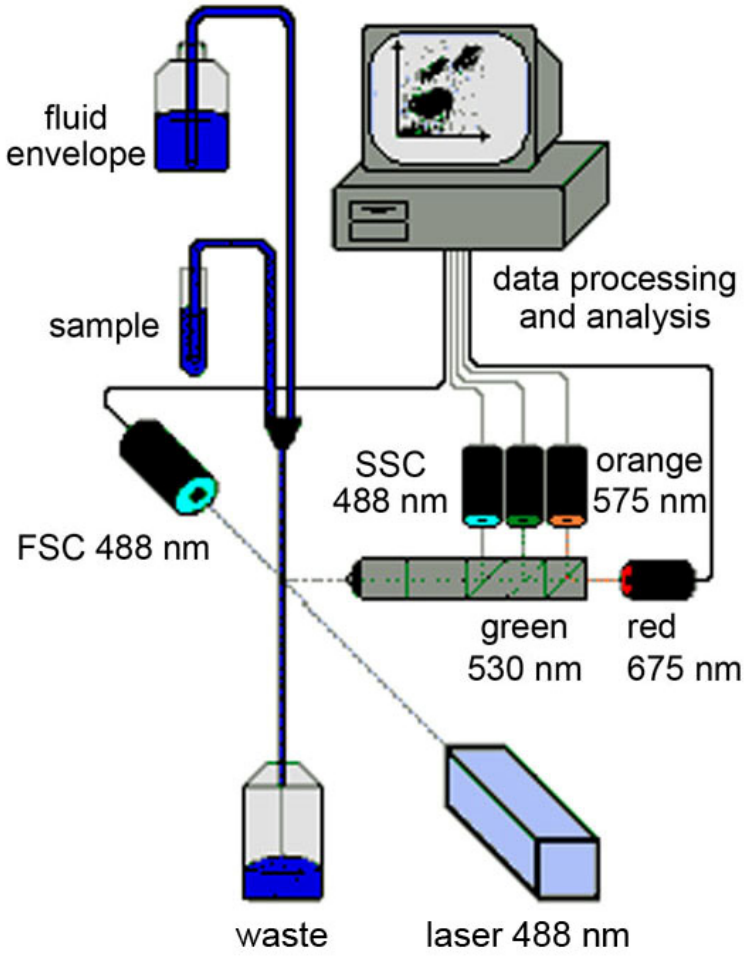


Figure 7. **Schematic diagram of flow cytometry**. The liquid system drives the analyzed cell from the sample tube (sample) through the laser beam. The sample beam is pulled apart by a sheath fluid (fluid envelope) to a very thin thread of liquid. The light scattered by the cell in the direction of the laser is detected by the FSC detector (forward scatter), the side scattered light (SSC, side scatter) at right angle to the laser beam passing through a lens system, different color filters and beam splitters is detected by the various detectors. The signals from the photomultiplier are amplified, and can be evaluated and displayed by a computer**.**

3.10.2 Assay procedure

For intracellular staining peripheral blood was collected in lithium-heparin tubes. 100 µl of blood was added to RPMI-1640 medium including brefeldin A (final concentration: 1 µg/ml) and incubated for 6 hours at 37°C. Then, erythrocytes were lysed by NH_4Cl. After washing with PBS/2% FCS cells were stained with FITC-conjugated monoclonal antibodies against the surface antigens CD3, CD4, CD8 and CD14 (15 min, room temperature), followed, after a washing step, by fixation with 100 µl 2% paraformaldehyde for 10 min at room temperature. After a wash the cells were incubated in 100 µl permeabilisation solution (0.1% saponin and 0.1% NaN_3 in PBS) together with 1 µl directly PE-conjugated anti-TNF-α, IL-2, IL-4, IL-5, IL-10, or IFN-γ antibodies for 15 min at room temperature. Followed by a wash with permeabilisation solution the cells were resuspended in PBS/2% FCS and fluorescence intensity was analyzed by flow cytometry (FACSCalibur, Becton-Dickinson, Heidelberg, Germany). For analysis regions were defined by forward scatter and side scatter as well as $CD3^+/CD4^+$- or $CD3^+/CD8^+$- lymphocyte and $CD14^+$- monocyte populations.

Cells incubated with PE stained non-specific IgG1 antibodies were analyzed in parallel to each measured approaches and measured as a negative control. A threshold was defined to discriminate the background fluorescence of the cells. Cells with a fluorescent signal that was below this threshold were considered negative, whereas cells with a fluorescent signal that was above this threshold were classified as positive or specifically stained. Ca. 1% to 2% of cells in the negative control were false positive. By subtracting this value from each measurement, the specifically stained cells or the proportion of cells expressing a defined protein are determined. The measurement was performed by using the software CellQuest (Becton Dickinson, Heidelberg, Germany) on a Macintosh computer. Three hundred cells were measured per second and about 5000 cells were analyzed.

3.11 Statistical analysis

Because the amount of the cytokines produced was different in each experiment, the effects on TNF-α production were normalized to unstimulated cells, which were set as one. Data were analysed by non-parametric methods when the measured variables were not normally distributed. Comparisons between groups were made with the Wilcoxon test. All values are reported as means ± SEM. Probability values of <0.05 were considered statistically significant. All data analyses were performed with commercially available statistical analysis software packages (SPSS 11.5; SPSS, Chicago, IL).

4 Results

4.1 gp130 and LIFR-β are co-expressed in PBMC

To determine whether gp130/LIFR-β is expressed in PBMC, I isolated and purified PBMC from whole blood from healthy volunteers, and cultured the PBMC in vitro for 24 hrs, and then tested gp130 and LIFR-β levels by Western blot analysis. The expression of CT-1 receptor subunits gp130 and LIFR are both detectable in PBMC (Fig. 8). The signal intensity of gp 130 is much stronger than that of LIFR-β. But never the less, a detectable signal for LIFR-β was obtained. More over, the application of CT-1 to the cells did not alter the expression of gp130 and LIFR-β.

Figure 8. **Representative expression of gp130 and LIF-β receptor in PBMC**. PBMC were plated on poly-l-lysine coated 100 mm dishes in RPMI 1640 + 10% FCS at densities of 10.000 cells/cm^2. After 24 hrs, cells were lysed and proteins were transferred to a 10% SDS-PAGE gel. Gp130 and LIF-β receptor expression was assayed by Western blotting using anit-gp130 and anti-LIFR-β antibodies showing a gp130 band at 60kDa and a LIFR-β band at 110kDa. Twenty-five µg of total protein was loaded per each lane. Actin served as loading control.

4.2 CT-1 activates PBMC

Activated PBMC are characterized by increased expression of certain cytokines or mediators of inflammation. It has been previously reported that STAT-1, STAT-3, ERK, P38 MAPK and JNK signaling pathways were evoked by various cytokines in PBMC (Dogusan, Hooghe et al. 2001; Sanchez-Margalet and Martin-Romero 2001; Zimmerer, Lehman et al. 2008; Broide, Scapa et al. 2009; Kim, Goo et al. 2009). However, the potent signaling pathways and cytokines or mediators involved in the CT-1 stimulation in PBMC are still unknown. To

address this problem, I stimulated PBMC by CT-1 at incremental concentrations of 10, 50, and 100 ng/ml for 15 min. Saline was used as a negative control. Afterwards, the phosphorylation of STAT-1, STAT-3, ERK, P38 MAPK and JNK in these cells was determined by Western blot analysis. After 15 min incubation with CT-1, a concentration dependent phosphorylation of ERK and P38 MAPK were found in PBMC compared with controls. However, there were no significant differences in phosphorylation of STAT-1, STAT-3 or JNK signaling between them (Fig. 9). These data showed that CT-1 activated the signaling pathway of ERK and P38 MAPK in PBMC, but not that of STAT-1, STAT-3 or JNK.

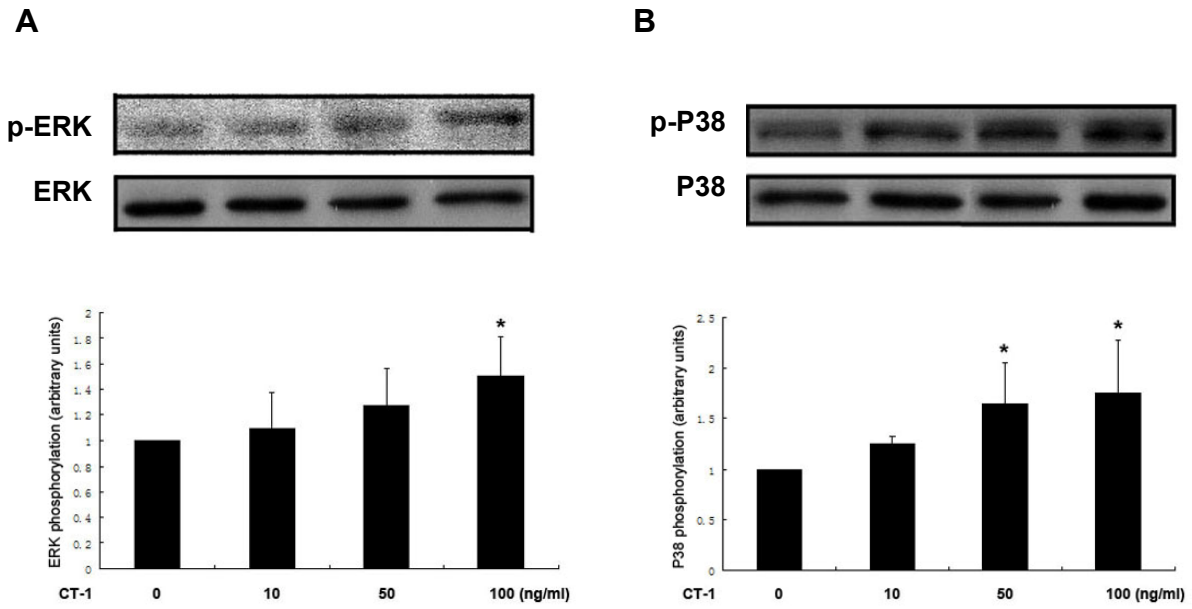


Figure 9. **Significant phosphorylation of ERK and P38 MAPK but not STAT-1, STAT-3 or JNK in PBMC after 15 min incubation with CT-1**. See next page for Legend.

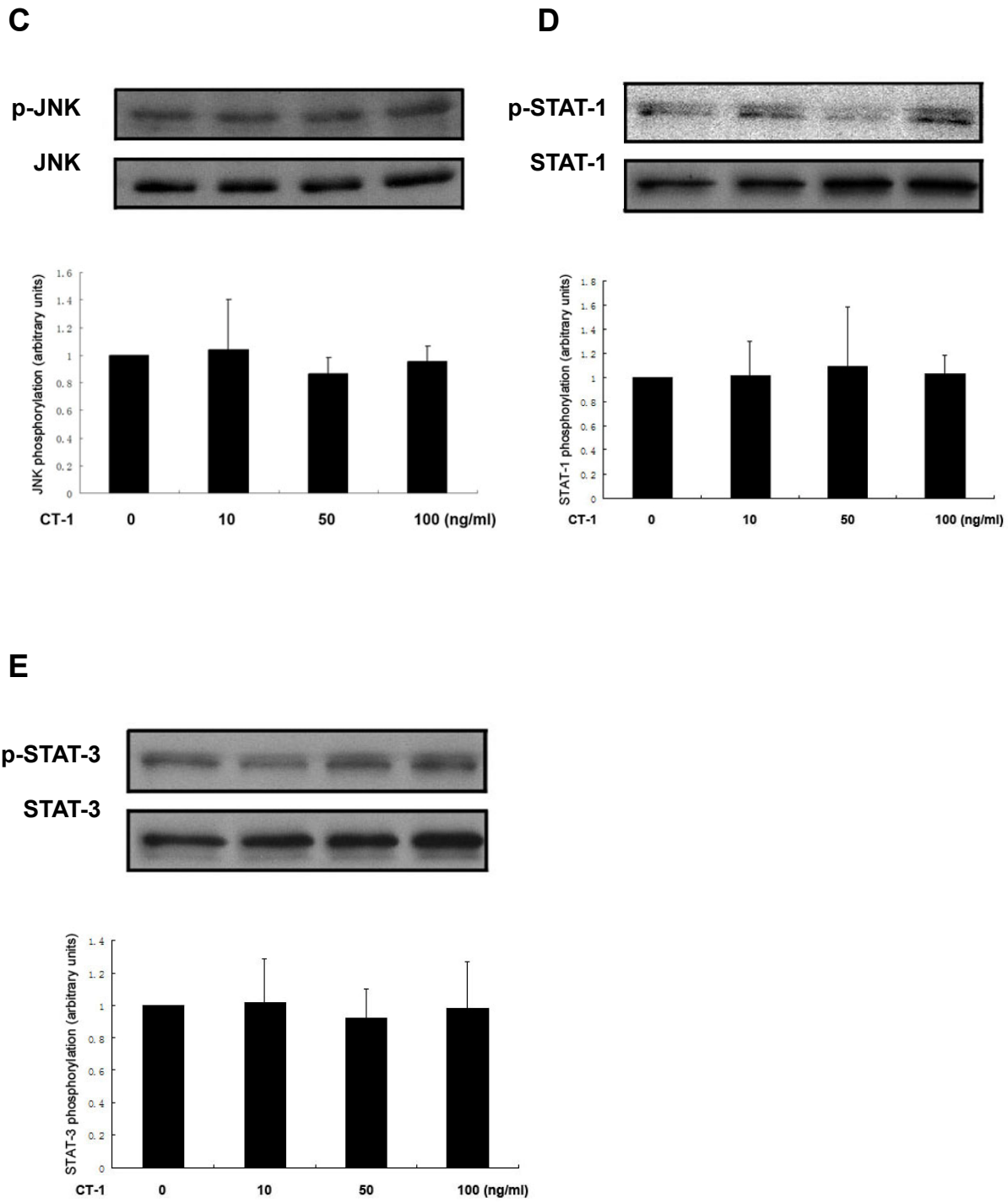


Figure 9. **Significant phosphorylation of ERK and P38 MAPK but not STAT-1, STAT-3 or JNK in PBMC after 15 min incubation with CT-1**. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with CT-1 at incremental concentrations or saline for 15 min and total cellular lysates were prepared. Lysates were resolved by SDS-PAGE and transferred to membranes by Western blot. The blots were detected with phosphor-specific STAT-1, STAT-3, ERK, P38 MAPK or JNK antibodies and reprobed with STAT-1, STAT-3, ERK, P38 MAPK or JNK antibodies, respectively. A: STAT-1 band at 89 kDa; B: STAT-3 band at 89 kDa; C: ERK band at 44/42 kDa; D: P38 MAPK band at 42 kDa; E: JNK band at 49 kDa. Twenty-five µg of total protein was loaded per lane. A significant phosphorylation of ERK and P38 MAPK

was observed, but no significant phosphorylation of STAT-1, STAT-3 and JNK. n=5, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

In the next sets of experiments I addressed whether CT-1 is able to induce the production of cytokines. For this purpose, human whole blood were incubated with CT-1 at a concentration of 50 ng/ml or saline as a control for 6 hrs in the presence of brefeldin A (10 µg/ml, an inhibitor of intracellular protein transport), and the intracellular accumulation of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α after CT-1 application was determined by flow cytometry.

After 6 hrs incubation with CT-1, a significant increased expression of TNF-α was observed in PBMC (6.4-fold) compared with control, but no significant changes were found in IL-2, IL-4, IL-5, IL-10 and IFN-γ expression (Fig. 10). These data indicate that under this experimental condition CT-1 induces only TNF-α production in PBMC.

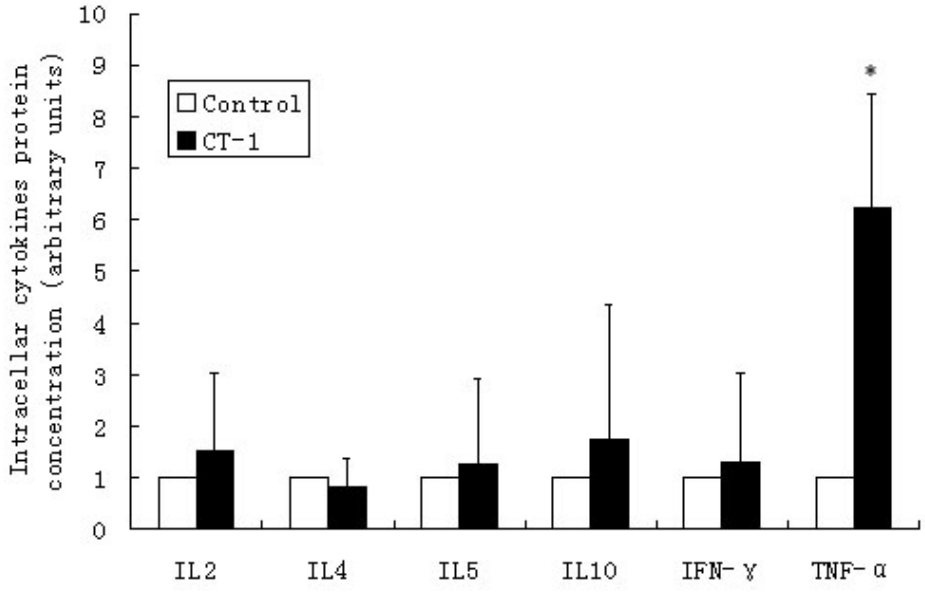


Figure 10. **Cytokines or mediators induced by CT-1 stimulation in PBMC.** Human blood was incubated with CT-1 at a concentration of 50 ng/ml for 6 hours in the presence of brefeldin A (10 µg/ml, an inhibitor of intracellular protein transport). Afterwards erythrocytes were lysed and cells were stained with a monoclonal antibody against IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α PE-conjugated. Results are expressed normalised to unstimlulated cells. A significant increased up to 6.4-fold expression of TNF-α was observed, but no significant changes were found in IL-2, IL-4, IL-5, IL-10, IFN-γ expression. n=6, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

4.3 CT-1 induces the expression of TNF-α at both mRNA and protein levels in PBMC

As was shown in **Section 4.2**, PBMC were demonstrated to be activated and initially observed to produce TNF-α by CT-1 sitimulation. I further validated whether CT-1 can promote the expression of TNF-α at both mRNA and protein levels in PBMC. In the first sets of experiments, cells were incubated with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for different periods of time various from 0.5 to 16 hrs. After the indicated time, RNA was isolated and TNF-α mRNA was determined by real-time-PCR. Compared with control, CT-1 induced a significantly upregulated expression of TNF-α mRNA in a concentration-dependent manner in PBMC after 1, 3 or 6 hrs incubation (all $P< 0.05$). Maximal TNF-α mRNA expression was found after 1 hour incubation. Afterwards mRNA expression of TNF-α declined. After 16 hrs incubation with CT-1, there was no significant difference in mRNA expression of TNF-α compared with control (Fig. 11).

Furthermore, to determine whether the CT-1 induced TNF-α expression is specified for CT-1 and whether this is receptor medicated, a CT-1 blocking antibody and an antibody blocking the gp130 (a part of the CT-1 receptor of gp130/LIFR-β heterodimer) were used. PBMC were stimulated with CT-1 at a concentration of 50 ng/ml with or without the presence of anti-CT-1 or anti-gp130 blocking antibody for 1 hour. Afterwards TNF-α mRNA level was determined by real-time-PCR. Cells incubated with medium alone served as a control. Without the presence of CT-1 blocking antibody, CT-1 induced a significantly upregulated mRNA expression of TNF-α compared with control. However in the presence of the CT-1 or gp130 blocking antibody, there is no significant difference in the TNF-α mRNA level after CT-1 stimulation compared with control (Fig. 12). These data indicate that the increase of TNFα expression in PBMC is specifically caused by CT-1 and this effect is receptor mediated via gp130 signaling.

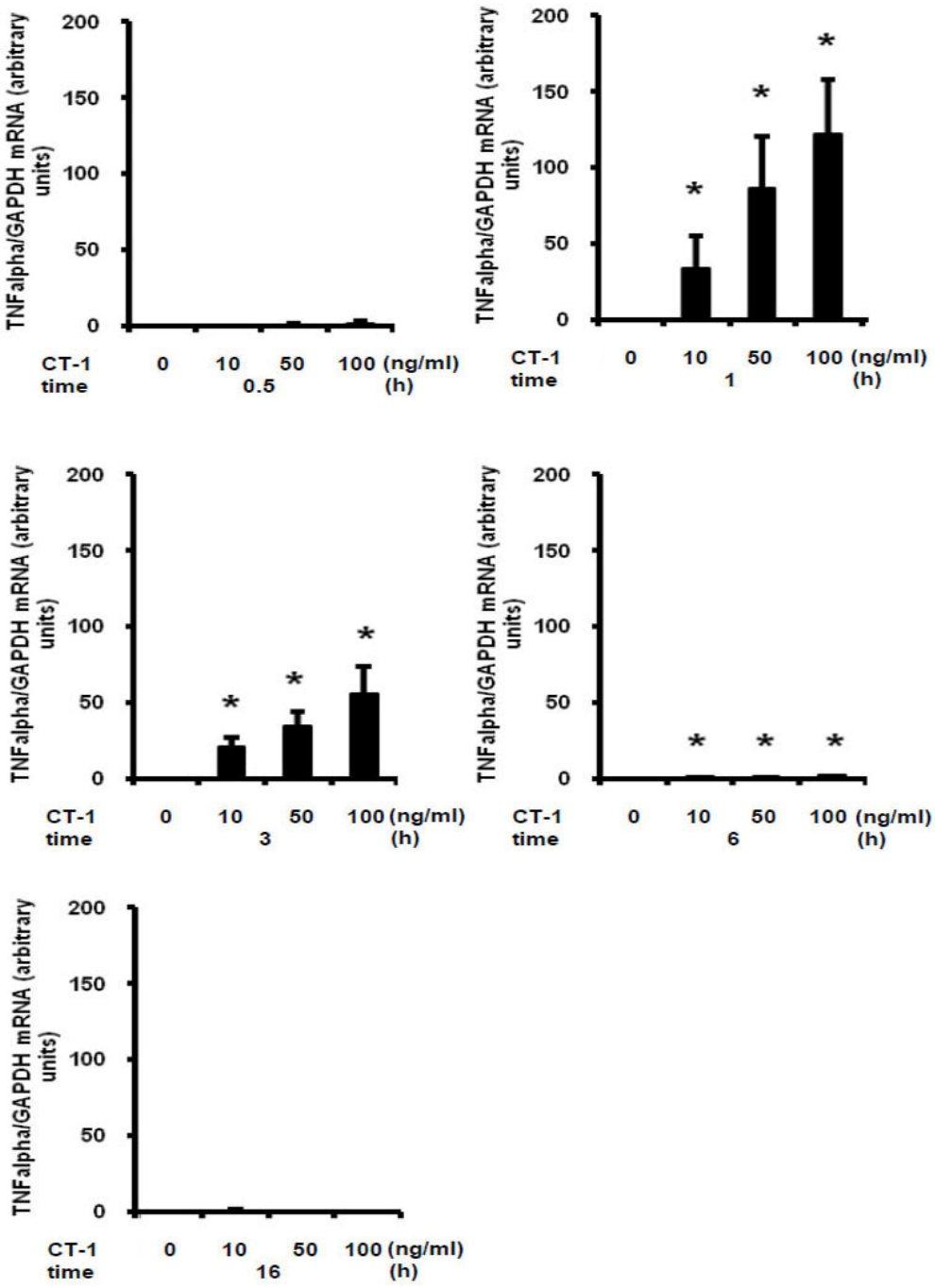


Figure 11. **Concentration- and time-dependent induction of TNF-α mRNA in PBMC after incubation with CT-1**. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for different periods of time. After the indicated time, mRNA was isolated and TNF-α mRNA was determined by real-time-PCR. All TNF-α mRNA expression data were normalized to GAPDH. n=8, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

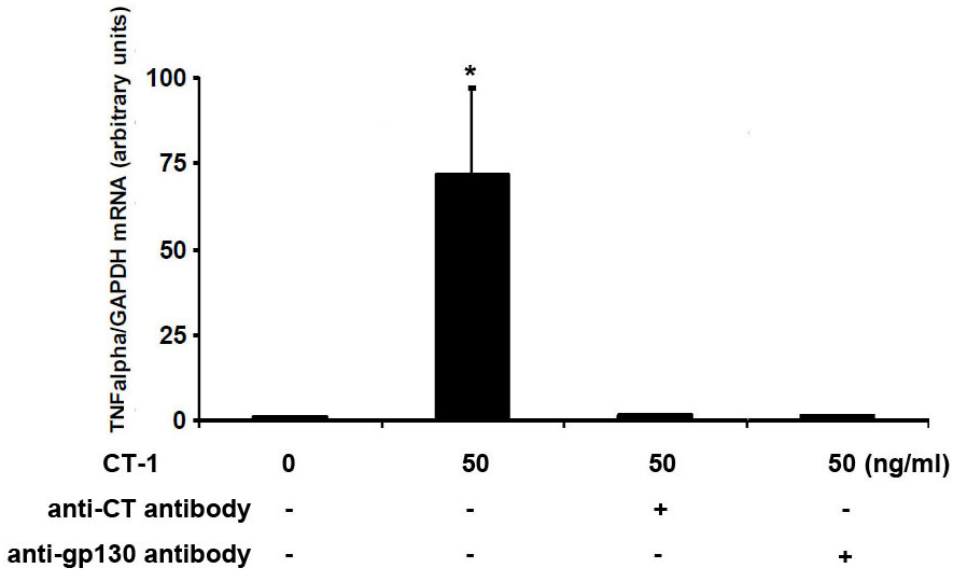


Figure 12. **Effect of anti-CT-1 or anti-gp130 blocking antibody on CT-1 induced TNF-α mRNA expression in human PBMC**. PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with or without anti-CT-1 or anti-gp130 blocking antibody. After 30 min CT-1 (50 ng/ml) was added for additional 1 h (0 ng/ml: control). Afterwards mRNA was isolated and TNF-α mRNA was determined by real-time-PCR. All TNF-α mRNA expression data were normalized to GAPDH. n=6, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

To futher investigate the protein expression of TNF-α in PBMC after CT-1 stimulation, cells were incubated with medium or CT-1 at a concentration of 10, 50 or 100 ng/ml for different time periods various from 1 to 24 hrs. After the indicated time supernatants were tested with a commercial available ELISA for the presence of TNF-α protein. As shown in Figure 13, CT-1 induced TNF-α protein production in the supernatant of PBMC in a concentration-dependent manner after 3, 6 or 16 hrs of incubation (all P< 0.05). Maximal TNF-α concentration was found after 3 to 6 hours. Afterwards TNF-α concentration declined. After 24 hrs incubation with CT-1, there was no significant difference in TNF-α concentration compared with control (Fig. 13). These results confirmed the previous flow cytometry data shown in Figure 10 that TNF-α protein production from PBMC was up-regulated after CT-1 stimulation, and also indicated that CT-1 caused only a transient TNF-α release in PBMC.

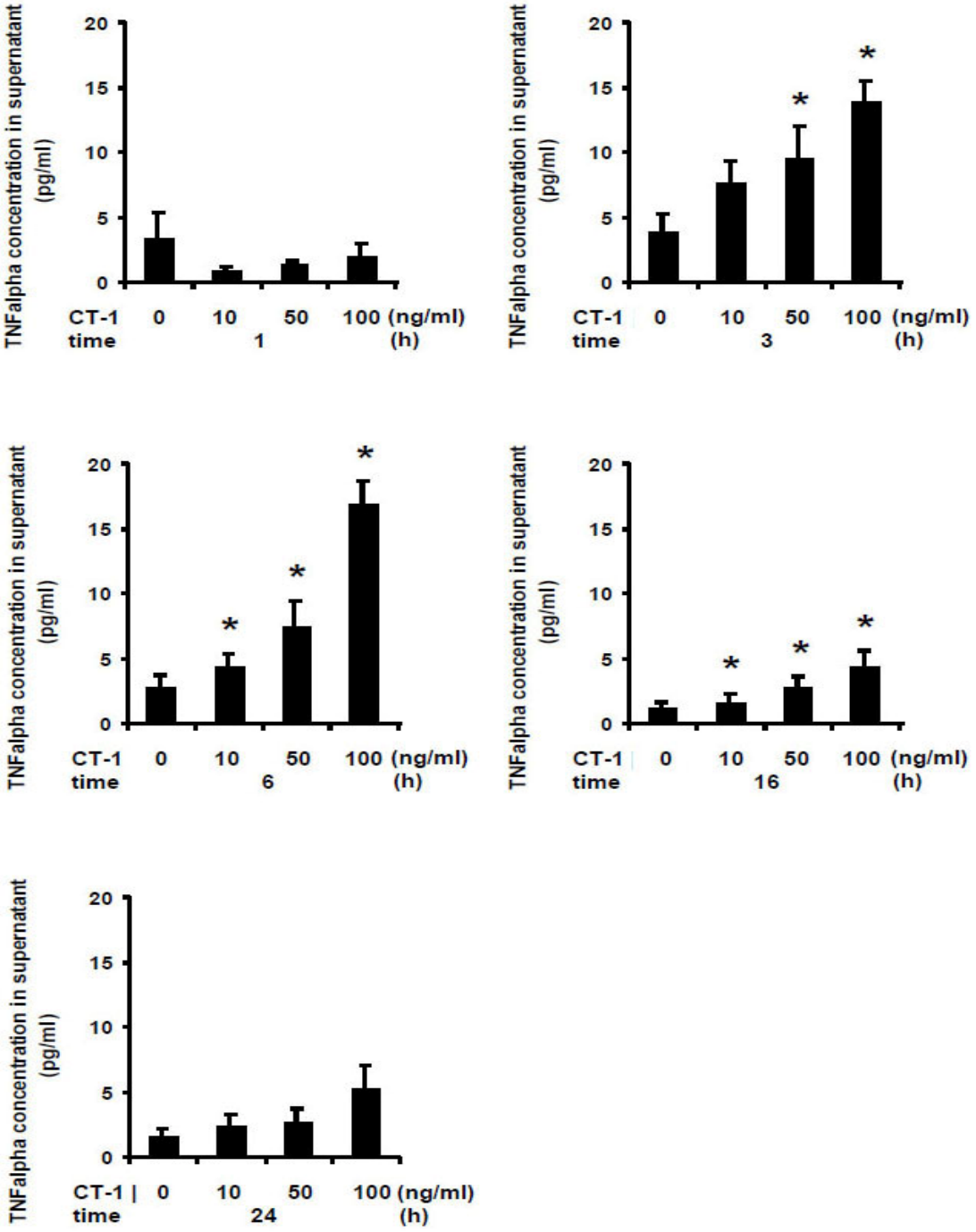


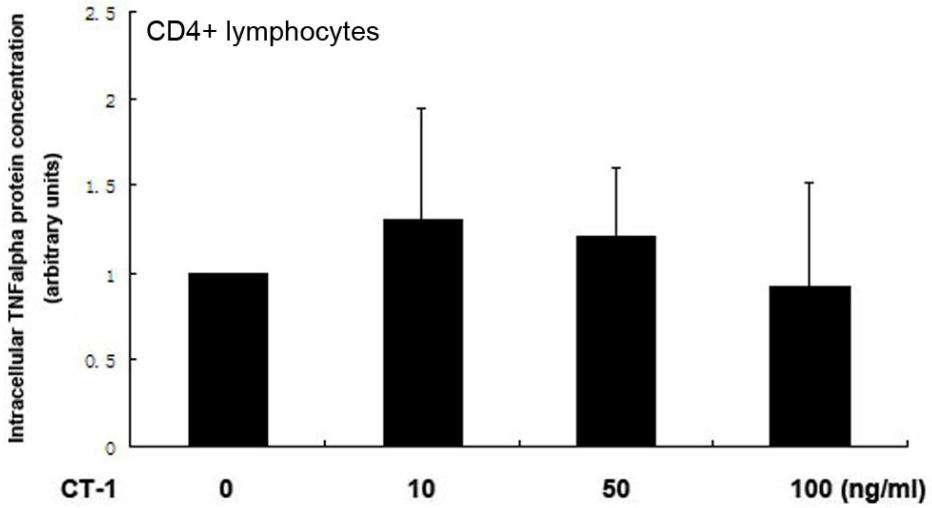
Figure 13. **Concentration- and time-dependent expression of TNF-α protein in the supernatant of PBMC after incubation with CT-1**. Human PBMC were isolated and plated on poly-l-lysine coated 6 wells plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for different time periods. After the indicated periods supernatants were tested with a commercial available ELISA for the presence of TNF-α protein. n=5, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

4.4 Monocytes but not lymphocytes are the PBMC subsets involved in the CT-1 induced TNF-α production

PBMC consist mainly of monocytes and lymphocytes. Although TNF-α is produced mainly by monocytes and macrophages, it is also produced by lymphocytes under certain circumstance. As shown in **Section 4.3**, CT-1 was demonstrated to be able to promote the expression of TNF-α in PBMC. I further identified which subpopulation of PBMC was involved in the production of TNF-α by CT-1 stimulation.

To overcome this issue, I incubated human blood with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for 6 hours in the presence of brefeldin A (10 µg/ml, an inhibitor of intracellular protein transport). Afterwards erythrocytes were lysed and cells were stained with a monoclonal antibody against CD3, CD4, CD8 or CD14 FITC-conjugated and against TNF-α PE-conjugated. Then, intracellular TNF-α protein in monocytes, $CD4^+$ and $CD8^+$ lymphocytes was determined by flow cytometry. Intracellular TNF-α protein determination in $CD4^+$ and $CD8^+$ lymphocytes did not show any effects of CT-1 on TNF-α expression (Fig. 14 A and B). In contrast, a concentration dependent significant increase of intracellular TNF-α after CT-1 application was found in monocytes (Fig. 14 C). These results showed that CT-1 induced increasing expression of TNF-α in PBMC was due to the activation of monocytes but not that of lymphocytes. In addition, these data together with that in **Section 4.3** indicated that culture conditions did not influence CT-1 induced TNF-α production in monocytes.

A



B

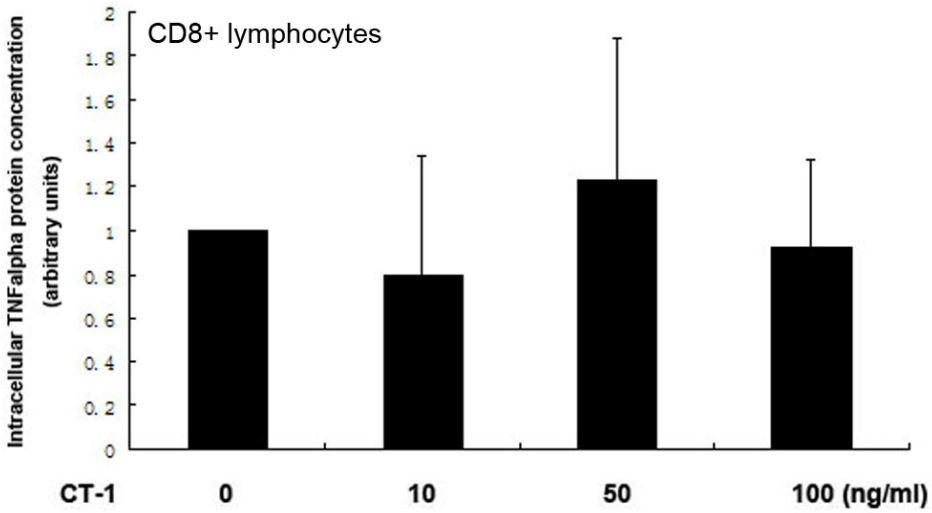


Figure 14. **Concentration-dependent induction of TNF-α intracellular protein expression in human peripheral blood monocytes after incubation with CT-1**. See next page for Legend.

C

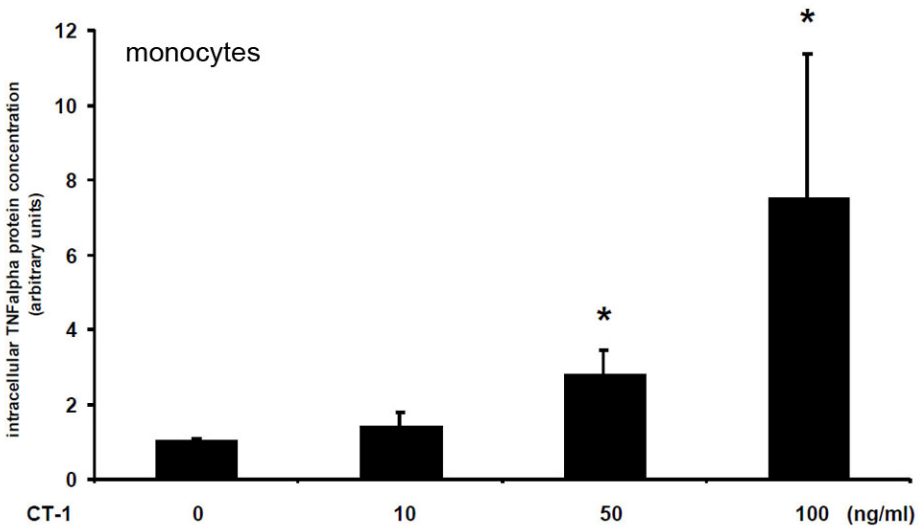


Figure 14. **Concentration-dependent induction of TNF-α intracellular protein expression in human peripheral blood monocytes after incubation with CT-1**. Human blood was incubated with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for 6 hours in the presence of brefeldin A (10 µg/ml, an inhibitor of intracellular protein transport). Afterwards erythrocytes were lysed and cells were stained with a monoclonal antibody against CD4, CD8 or CD14 FITC-conjugated and against TNF-α PE-conjugated. Monocytes or lymphocytes were gated and results are expressed normalised to unstimlulated cells. A: $CD4^+$ lymphocytes; B: $CD8^+$ lymphocytes; C: $CD14^+$ monocytes. n=11, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

4.5 The effect of CT-1 on TNF-α expression in PBMC is dependent on mRNA synthesis and intracellular protein transport

With the next experiments I addressed the question whether TNF-α protein expression is dependent on mRNA synthesis or intracellular protein transport. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plates in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with actinomycin D (inhibitor of mRNA synthesis) or brefeldin A (inhibitor of intracellular protein transport). After 30 min CT-1 (50 ng/ml) or saline was added to the cells for additional 3 hrs. Supernatants were tested with a specific ELISA for the presence of TNF-α protein. As shown in Figure 15, both inhibition of mRNA synthesis and inhibition of intracellular protein transport were

able to abolish CT-1 induced TNF-α protein induction. These results showed that CT-1 was responsible for new protein synthesis of TNF-α protein in PBMC, and that TNF-α protein was secreted into the supernatant actively.

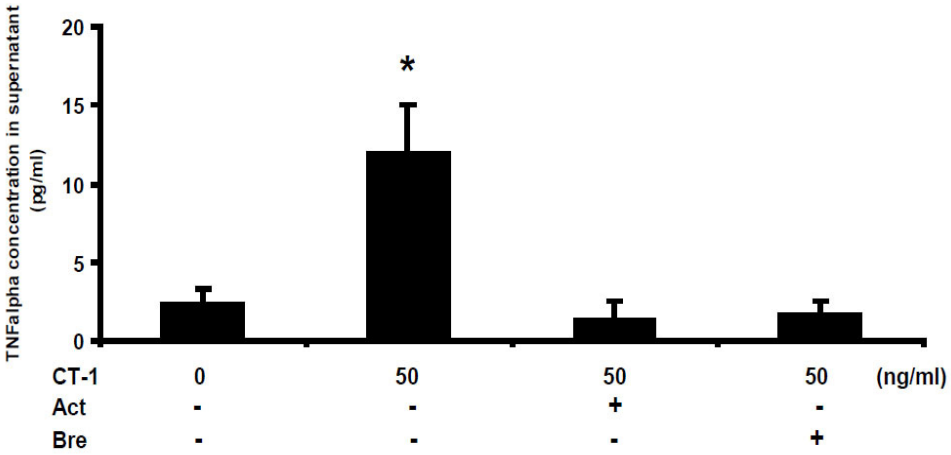


Figure 15. **CT-1 induced TNF-α protein expression in the supernatant of PBMC was abolished by actinomycin D or brefeldin A**. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with actinomycin D or brefeldin A. After 30 min CT-1 (50 ng/ml) was added for additional 3 hrs (0 ng/ml: control). Supernatants were tested with a specific ELISA for the presence of TNF-α protein. Act: actinomycin D (5 µg/ml), inhibits mRNA transcription, Bre: brefeldin A (10 µg/ml), inhibits intracellular protein transport. n=6, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

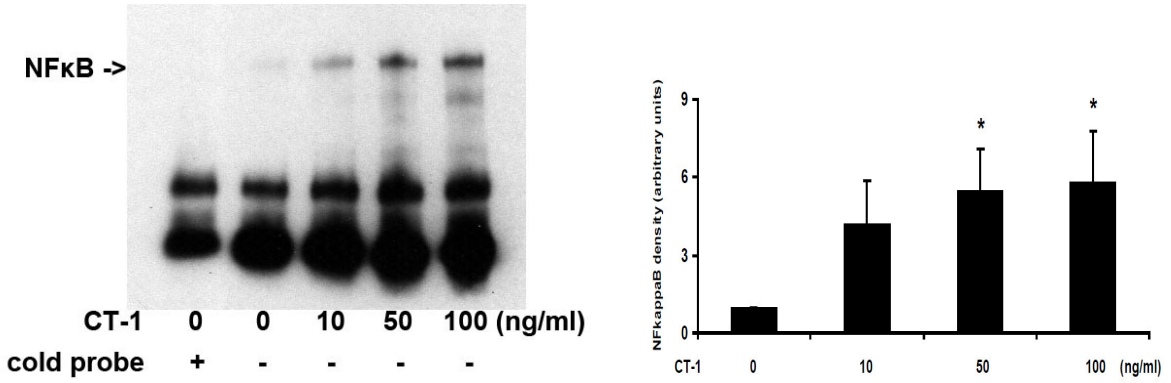
4.6 CT-1 induces NFκB (nuclear factor kappa B) translocation into the nucleus in PBMC

With the next experiments I addressed the underlying signaling pathway of CT-1 induced TNF-α expression in PBMC. It has been previously shown that transcription factors STAT-1, STAT-3, NFκB, activator protein 1 (AP-1) and specificity protein 1 (SP1) were involved in the signaling pathways of various cytokines in PBMC (Madden, Vince et al.; Nicolet, Surfus et al. 1998; Sanchez-Margalet and Martin-Romero 2001; Flores, Duran et al. 2003; Kooijman, Coppens et al. 2006; Zimmerer, Lehman et al. 2008). However, the transcription factors involved in the CT-1 activated signaling pathways in PBMC are still unknown. To overcome this problem, human PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a

concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for 25 min. Nuclear extracts were subjected to an electrophoretic mobility shift assay, then STAT-1/DNA, STAT-3/DNA, NFκB/DNA, AP-1/DNA and SP1/DNA complexes were visualized by a chemiluminescent imaging system.

As shown in Figure 16 A, CT-1 caused a concentration-dependent NFκB translocation to the nucleus determined by EMSA reaching maximal translocation by CT-1 sitimulation at a concentration of 100 ng/ml. However, no significant translocation to the nucleus was found for STAT-1, STAT-3, AP-1 and SP1 (Fig. 16 B-E). These data suggest that PBMC exhibit a CT-1 induced transcription factor activation in which NFκB is involved.

A



B

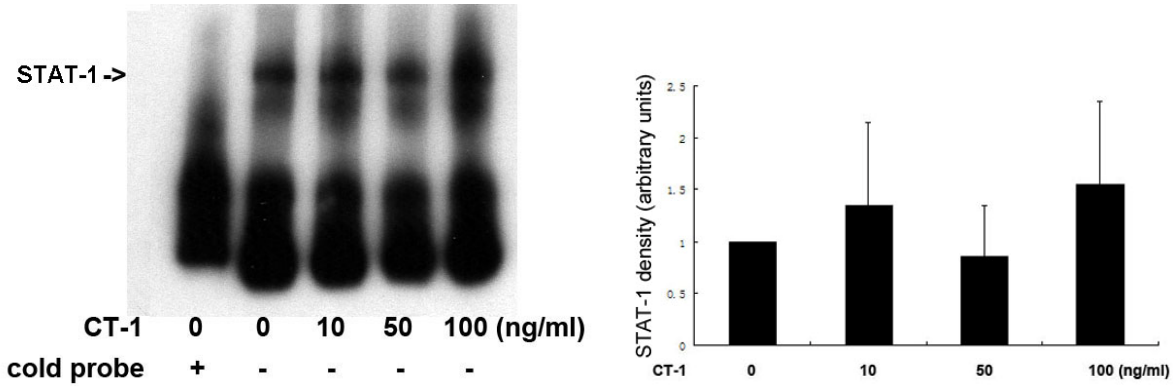
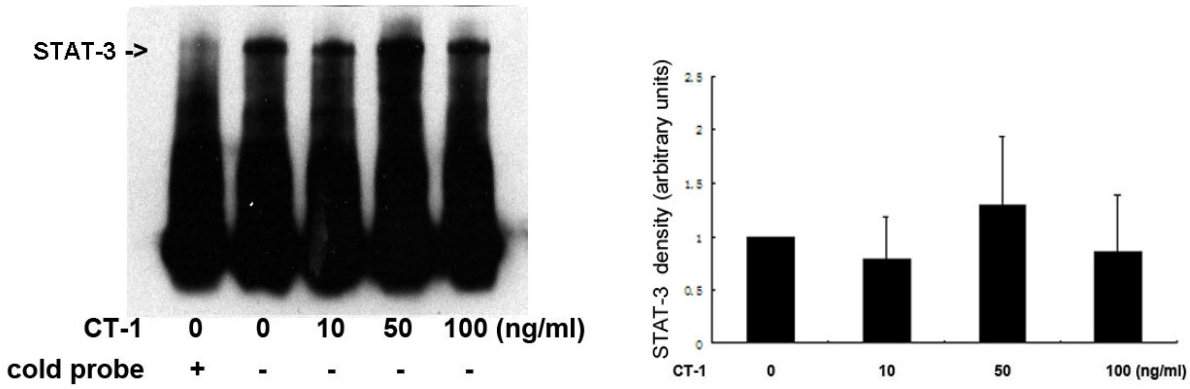


Figure 16. **CT-1 induced transcription factors activity measured by EMSA**. See next page for Legend.

C



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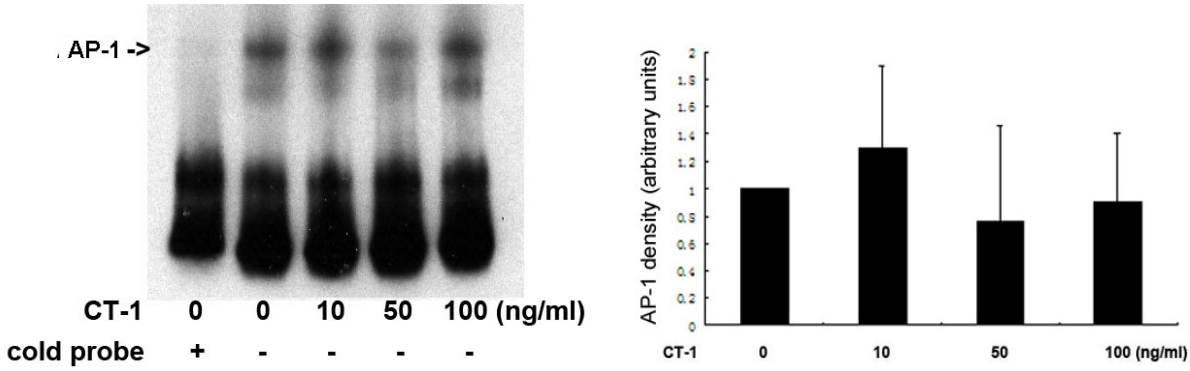

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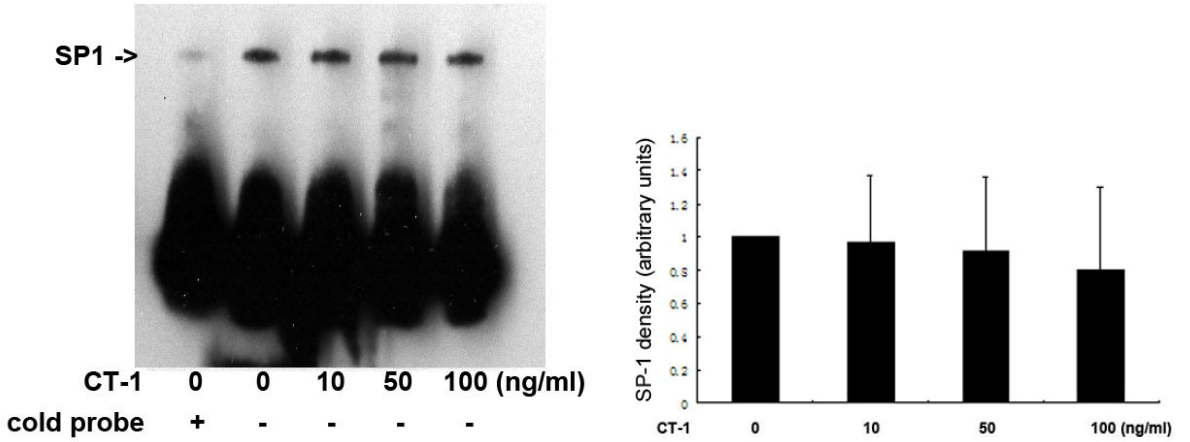

Figure 16. **CT-1 induced transcription factors activity measured by EMSA**. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with medium alone or CT-1 at a concentration of 10, 50 or 100 ng/ml for 25 min. Nuclear

extracts were subjected to an electrophoretic mobility shift assay, then STAT-1/DNA, STAT-3/DNA, NFκB/DNA, AP-1/DNA and SP/DNA complexes were visualized by a chemiluminescent imaging system. Bands corresponding to the transcription factors activity were quantified by densitometry and expressed in arbitrary units and normalized to unstimulated PBMC. A: STAT-1; B: STAT-3; C: NFκB; D: AP-1; E: SP1. n=7, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

4.7 CT-1 induced NFκB translocation into the nucleus in PBMC is mediated by IκB degradation

NFκB is a rapidly acting primary transcription factor found in all cell types. It is involved in cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immune response. In unstimulated cells NFκB dimers are sequestered inactively in the cytoplasm by a protein complex called IκB. IκB inactivates NFκB by masking the nuclear localisation signals (NLS). Activation of NFκB occurs via degradation of IκB, a process that is initiated by its phosphorylation by IκB kinase (IKK). Phosphorylated IκB becomes dissociated from NFκB, unmasking the NLS. Phosphorylation also results in IκB ubiquitination and targeting to the proteasome. Then NFκB can translocate into the nucleus and regulate gene expression. NFκB also induces the expression of IκB forming a negative feedback loop.

To test whether CT-1 induced NFκB translocation to the nucleus in PBMC is mediated by IκB degradation, I stimulated PBMC by CT-1 at a concentration of 50 ng/ml or saline as a control for 5 min and then determined the concentration of IκB in these cells by Western blotting. After 5 min incubation with CT-1, a significant decrease of IκB was found in PBMC compared with sham controls ($P< 0.05$) (Fig. 17). These data showed that CT-1 induced an up to 5 min instantly degradation of IκB in PBMC, indicating that CT-1 caused NFκB activation by IκB degradation.

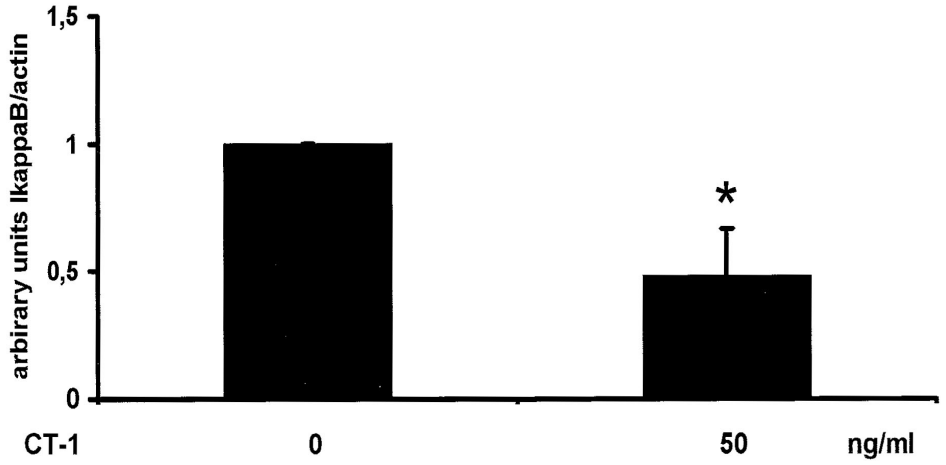

Figure 17. **CT-1 induced IκB degradation measured by Western blot**. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with medium alone or CT-1 at a concentration of 50 ng/ml for 5 min. Afterwards PBMC were lysed. Lysates were resolved by SDS-PAGE and transferred to membranes by Western blot. The blots were detected with an anti-IκB antibody. Twenty five µg of total protein was loaded per lane. A significant decrease of IκB was observed after CT-1 stimulation. n=5, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

4.8 CT-1 induces TNF-α expression in PBMC via the NFκB pathway

In the next sets of experiments I addressed the question whether NFκB translocation was responsible for CT-1 induced TNF-α expression in PBMC. Human PBMC were isolated and plated on poly-l-lysine coated 6 well plates in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with parthenolide (50 µM, an inhibitor of NFκB activation). After 30 min CT-1 (50 ng/ml) was added for additional 25 min (0 ng/ml: control). Human umbilical vein endothelial cells (HUVEC) stimulated with TNF-α served as a control. Nuclear extracts were subjected to EMSA determination and NFκB/DNA complex was visualized by a chemiluminescent imaging system.

As shown in Figure 18, unstimulated cells did not show significant NFκB protein in the nucleus, whereas CT-1 caused translocation of NFκB into the nucleus. Parthenolide was able to inhibit NFκB translocation into the nucleus.

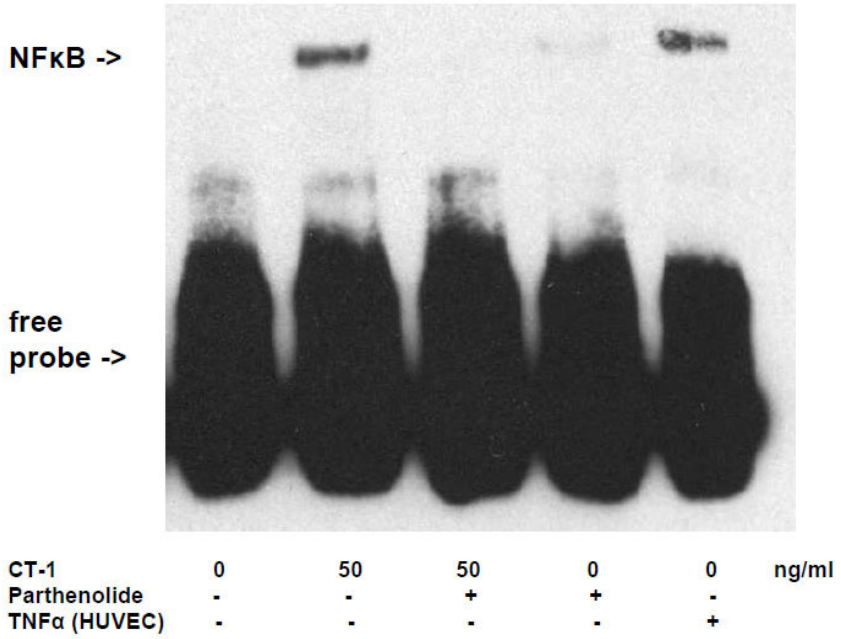

Figure 18. **Representative EMSA of CT-1 induced NFκB translocation into the nucleus in human PBMC was inhibited by parthenolide**. Human PBMC were isolated and plated on poly-l-lysine coated 6 wells plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with parthenolide (50 μM, an inhibitor of NFκB activation). After 30 min CT-1 (50 ng/ml) was added for additional 25 min (0 ng/ml: control). Nuclear extracts were subjected to an electrophoretic mobility shift assay and NFκB/DNA complex was visualized by a chemiluminescent imaging system. Representative EMSA of CT-1 induced NFκB activity in PBMC was inhibited by parthenolide. HUVEC cells stimulated with TNF-α were used as positive control. n=6, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

To further investigate the pathway responsible for NFκB translocation into the nucleus is essential for CT-1 induced expression of TNF-α in PBMC, I generate an experiment to compare the expression of TNF-α in PBMC under a condition with or without the presence of parthenolide (an inhibitor of NFκB activation), SB203580 (an inhibitor of P38 MAPK activation) and PD98059 (an inhibitor of ERK activation). PBMC were isolated and plated on poly-l-lysine coated 6 well plates in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with parthenolide (50 μM), SB203580 (5 μM) or PD98059 (30 μM) respectively. After 30 min CT-1 (50 ng/ml) or saline (use as control) was added for additional 1 hour (for real-time-PCR determination) or 3 hrs (for ELISA mesurement). Afterwards RNA was isolated and TNF-α mRNA

was determined by real-time-PCR, and the supernatants were tested with a specific ELISA for the presence of TNF-α protein.

As shown in Figure 19 and 20, CT-1 caused a significant elevated expression of TNF-α at both mRNA and protein level in PBMC compared with control. In the presence of parthenolide the CT-1-induced expression of TNF-α decreased significantly to a level that was not significantly different from the control. In contrast, neither SB203580 nor PD98059 had an effect on the CT-1-induced expression of TNF-α. In the presence of SB203580 or PD98059 there is still a significant difference in the expression of TNF-α between CT-1 stimulation and the control. These data showed that CT-1 induced TNF-α expression in PBMC was able to completely inhibited by parthenolide at both protein and mRNA level, but not by SB203580 or PD98059, suggesting that CT-1 induced TNF-α expression is due to NFκB activation but not depended on P38 MAPK or ERK activation.

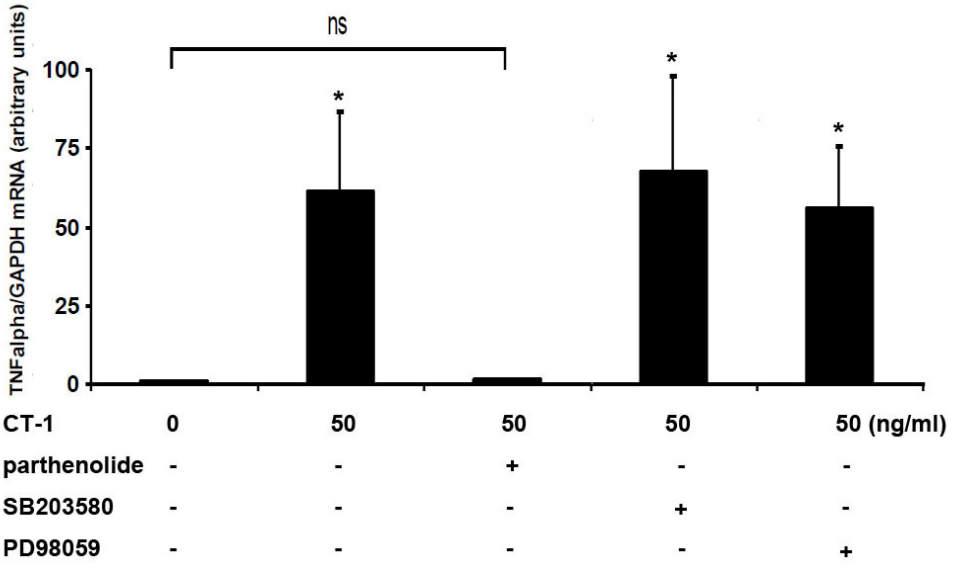

Figure 19. **Effect of signal inhibitors on CT-1 induced TNF-α mRNA expression in human PBMC**. PBMC were isolated and plated on poly-l-lysine coated 6 wells plate in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with parthenolide (50 µM, an inhibitor of NFκB activation), SB203580 (5 µM, an inhibitor of P38 MAPK activation) or PD98059 (30 µM, an inhibitor of ERK activation). After 30 min CT-1 (50 ng/ml) was added for additional 1 h (0 ng/ml: control). Afterwards RNA was isolated and TNF-α mRNA was determined by real-time-PCR. All TNF-α mRNA expression data were normalized to GAPDH. n=6, data are expressed as mean ± SEM. *P<0.05 compared with unstimulated cells.

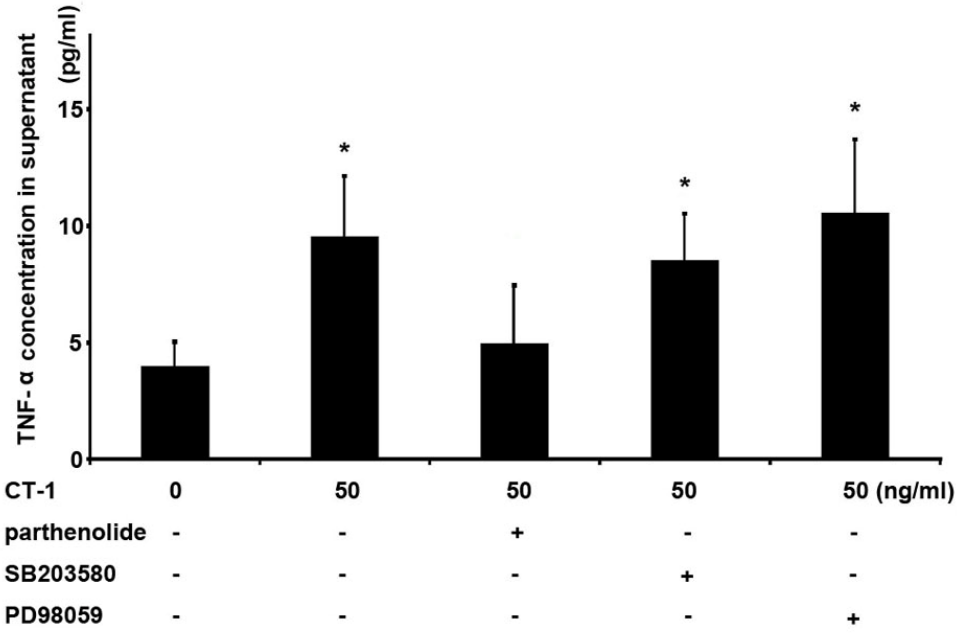

Figure 20. **Effect of signal inhibitors on CT-1 induced TNF-α protein expression in human PBMC**. PBMC were isolated and plated on poly-l-lysine coated 6 well plates in RPMI 1640 + 10% FCS at a concentration of 10.000 cells/cm^2. After 24 hrs, cells were incubated with parthenolide (50 µM, an inhibitor of NFκB activation), SB203580 (5 µM, an inhibitor of P38 MAPK activation) or PD98059 (30 µM, an inhibitor of ERK activation). After 30 min CT-1 (50 ng/ml) was added for additional 3 h (0 ng/ml: control). Afterwards supernatants were tested with a specific ELISA for the presence of TNF-α protein. n=5, data are expressed as mean ± SEM and normalized to unstimulated cells. *P<0.05 compared with unstimulated cells.

To further examine the effect of parthenolide on CT-1 induced TNF-α protein intracellular expression in human peripheral blood monocytes, human blood was incubated with brefeldin A (10 µg/ml, an inhibitor of intracellular protein transport) and parthenolide (50 µM, an inhibitor of NFκB activation). After 30 min CT-1 (50 ng/ml) was added for additional 6 hrs (0 ng/ml: control). Afterwards erythrocytes were lysed and cells were stained with a monoclonal antibody against CD14 FITC-conjugated and against TNF-α PE-conjugated. Using flow cytometry monocytes showed an increase of intracellular TNF-α after CT-1 application which could be completely inhibited by parthenolide (Fig. 21). Parthenolide alone did not show a significant effect on TNF-α expression in unstimulated cells. These results show that CT-1 induced TNF-α in PBCM independent of culture conditions and independent of determination methodes and NFκB seems to be

essential for the effect of CT-1 on TNF-α induction in PBMC.

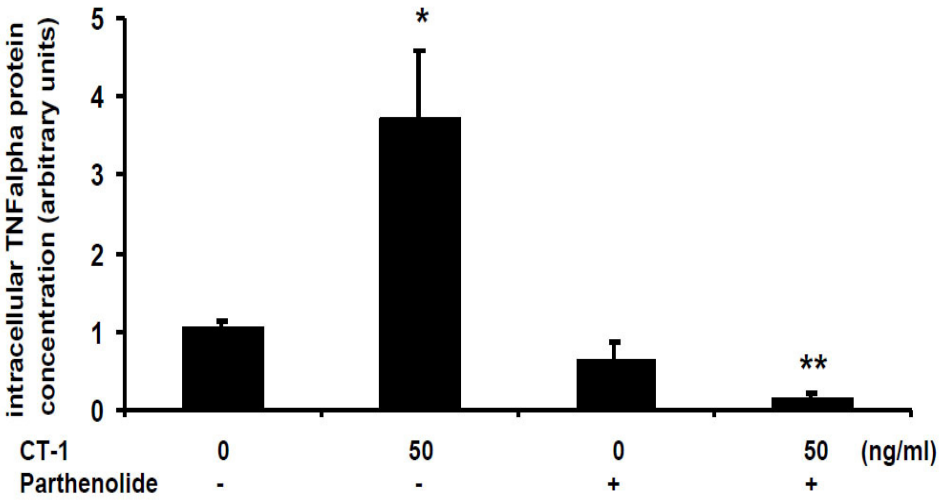

Figure 21. **Effect of parthenolide on CT-1 induced TNF-α protein intracellular expression in human peripheral blood monocytes**. Human blood was incubated with brefeldin A (10 µg/ml, an inhibitor of intracellular protein transport) and parthenolide (50 µM, an inhibitor of NFκB activation). After 30 min CT-1 (50 ng/ml) was added for additional 6 h (0 ng/ml: control). Afterwards erythrocytes were lysed and cells were stained with a monoclonal antibody against CD14 FITC-conjugated and against TNFα PE-conjugated. Monocytes were gated and results are expressed normalised to unstimlulated monocytes. n=6, data are expressed as mean±SEM. *P<0.05 compared with unstimulated cells, **P<0.05 compared with cells stimulated with 50 ng/ml CT-1.

5 Discussion

In CHF, activation of the immune system occurs, which results in the production and release of proinflammatory cytokines, activation of the complement system, and production of autoantibodies. All of these pathophysiological processes can cause a progression of CHF. Recent experimental evidence suggests a pathway of cytokine release by cardiac cells, both myocytes and nonmyocytes, in response to hemodynamic stress. Following hemodynamic load mice developed cardiac dysfunction in association with a wide array of activation of proinflammatory cytokines and a decrease in anti-inflammatory cytokines in cardiac cells with little or no inflammatory infiltrates, indicating that the hemodynamic load alone is capable of producing cardiac inflammation (Celis, Torre-Martinez et al. 2008). Although in that study the actions of cytokines released by cardiac cells are locally limited in the heart, from these findings I was enlightened to form an idea that substances released by cardiac cells might also be released into the circulation and exert endocrine or exocrine effects to initiate or participate in the inflammatory response in heart failure.

To verify this idea, I looked for a potential substance which was exclusively produced by the failing cardiac cells and capable of inducing an inflammatory response. The observations that CT-1, produced predominantly by heart, is increasingly expressed and secreted via the coronary sinus into the peripheral circulation in CHF, and that CT-1 is a strong acute-phase mediator to induce a dose-dependent production of several acute phase proteins (Robledo, Guillet et al. 1997), suggest that CT-1 could play an important role in the regulation of the inflammatory response seen in CHF, I therefore focus my interest of concern on CT-1 as a candidate of potential substance for study.

Because in CHF many proinflammatory cytokines are elevated and PBMC are activated, it is not easy to study the effect of a single cytokine in PBMC from patients with CHF. To elucidate a potential effect of CT-1 in CHF on PBMC, I used an in vitro cell culture model of PBMC from healthy volunteers and stimulated them with CT-1 in various concentrations and various experimental condistions.

This study is the first to demonstrate that CT-1 activates PBMC via gp130/ LIFR-β receptor and induces TNF-α production via NFκB signaling pathway in monocytes but not in lymphocytes. The results of this work reveal a new mechanism of immune activation in heart failure and elucidate a potential role of CT-1 in the pathophysiology of CHF. In addition, the data illustrate that heart is not only just a target of inflammation in CHF, but also seems to play a role in the activation of the proinflammatory state by secreting CT-1 into the circulation.

5.1 gp130 and LIFR-β are co-expressed in PBMC

Studies on the activities of CT-1 and its receptor have shown that, in several in vitro assays, CT-1 acts similarly to cytokines of the IL-6 family, and that CT-1 could bind to and induce biological responses via LIFR complex (gp130/LIFR-β heterodimer) (Pennica, Shaw et al. 1995).

Although gp130 is ubiquitously expressed, the number of cells that respond to a certain IL-6-type cytokine is limited, since the expression of the other receptor subunits is more restricted and tightly regulated. To the best of my knowledge, in the present study, I found for the first time that CT-1 receptor subunits gp130 and LIFR which were requested to build a heterodimer for CT-1 action were both expressed on PBMC, suggesting that CT-1 might have an effect on these cells. I therefore considered the PBMC a suitable experimental model to further study CT-1 action.

In addition, I also observed that the application of CT-1 to PBMC did not alter the expression of gp130 and LIFR-β, which would ensure a high affinity and efficacy to create ligand-receptor complex and further initiate a response.

5.2 CT-1 activates PBMC

Activated PBMC are characterized by activated signaling pathway and increased expression of several cytokines or mediators of inflammation. The present study showed an activation of signaling pathways and production of cytokine of TNF-α induced by CT-1 in PBMC. These data confirmed that CT-1 is

able to directly activate PBMC in the absence of any additional stimuli. This activation exhibits a distinct profile of signaling pathways activity and cytokines expression.

5.2.1 CT-1 activates signaling pathways in PBMC

In the present study, I determined the CT-1 induced signaling pathways in PBMC. I found significant phosphorylation of ERK and P38 MAPK in PBMC compared with controls after incubation with CT-1. These results demonstrate that CT-1 can activate the ERK and P38 MAPK signaling pathways in PBMC, which is in good aggreement with previous studies in PBMC that show active pathways of ERK and P38 MAPK after stimulation of various stimuli, e.g. lipopolysaccharide (LPS), insulin-like growth factor-1, rhizoctonia bataticola lectin, prolactin, vibrio vulnificus or 5-hydroxytryptamine (Vanlandschoot, Roobrouck et al. 2002; Cloëz-Tayarani, Kayyali et al. 2004; Kooijman, Coppens et al. 2006; Kim, Goo et al. 2009; Pujari, Nagre et al. 2010). In contrast to previous studies that showed an active STAT-1, STAT-3 or JNK signaling in PBMC (Dogusan, Hooghe et al. 2001; Sanchez-Margalet and Martin-Romero 2001; Kooijman, Coppens et al. 2006; Zimmerer, Lehman et al. 2008), the results presented here demonstrate no activation of STAT-1, STAT-3 or JNK pathway in PBMC after CT-1 stimulation. Therefore, these data showed that the signaling induced by CT-1 exhibits a distinct pattern of activation in ERK and P38 MAPK signaling but not in STAT-1, STAT-3 or JNK, which will provide a basis for future studies on the specific effects via these distinct signaling pathways in PBMC.

As described in the **Introduction section**, the signaling pathway downstream from gp130 consists of three distinct pathways. It is likely that CT-1 achieves its effects by certain pathway(s) depending on the particular effect examined or the cell type studied. While several studies agree on the effects mediated by the MAPK pathway, our knowledge about the actions mediated by the JAK/STAT pathway are contrasting. MAPK pathways might be involved in CT-1's capacity to promote the survival of terminally differentiated cell types. These effects were significantly blocked after treatment with PD98059, a MAPK kinase (MEK) 1

inhibitor, that, in contrast, did not block the activation of STAT3 or the hypertrophic response of cardiomyocytes following stimulation of CT-1 (Sheng, Knowlton et al. 1997). Recent studies have confirmed the involvement of the p42/44 MAPK pathway in protective effects of CT-1 (Brar, Stephanou et al. 2001; Liao, Brar et al. 2002; Railson, Liao et al. 2002). On the other hand, the role of the JAK/STAT pathway is controversial. The majority of studies suppose that the activation of STAT3 promotes myocardial hypertrophy (Sheng, Knowlton et al. 1997; Kunisada, Tone et al. 1998; Kunisada, Negoro et al. 2000; Railson, Liao et al. 2002); however, according to a recent study, the major pathway responsible for the hypertrophic response to CT-1 is not the JAK/STAT3 pathway nor the MEK/ERK1/2 pathway, but the pathway, MEK5/ERK5 (Takahashi, Saito et al. 2005). Moreover, the JAK/STAT pathway is implicated in beneficial mechanism, such as, transducing protective signals against doxorubicin-induced (Kunisada, Negoro et al. 2000) and postpartum cardiomyopathy (Hilfiker-Kleiner, Kaminski et al. 2007), protecting cardiomyocytes from ischemic (Negoro, Kunisada et al. 2000) and oxidative stress (Negoro, Kunisada et al. 2001), promoting myocardial angiogenesis and, consequently, tissue oxygenation during reperfusion, and controlling interstitial collagen metabolism with a reduction in cardiac fibrosis (Hilfiker-Kleiner, Hilfiker et al. 2004). Concerning the PI3K/Akt pathway, it promotes, as does the p42/44 MAPK pathway, cardiac myocyte survival against doxorubicininduced apoptosis (Negoro, Oh et al. 2001). It is interesting to note that, regarding cardioprotection against non-ischemic stimuli (Lopez, Diez et al. 2005) and re-oxygenation (Brar, Stephanou et al. 2001) CT-1 mediated, neither activation of the MEK1, p42/44 pathway nor PI3K/Akt alone is sufficient. Therefore MAPK and PI3K/Akt could cooperate in CT-1 mediated pro-survival effects (Kuwahara, Saito et al. 2000; Brar, Stephanou et al. 2001; Lopez, Diez et al. 2005).

5.2.2 CT-1 promotes cytokines production from PBMC

PBMC are a very important part of our immune system. PBMC consist mainly of monocytes and lymphocytes. Several important cytokines that act as mediators and regulators of immune processes are well known to be produced by these

cells. In response to pathogens and stimulation of specific cell-surface receptors, PBMC with heterogeneous multifunctional cellular population are activated and produce cytokines, e.g. TNF-α, IL-2, IL-4, IL-5, IL-10 and IFN-γ.

Our result showed that CT-1 activated PBMC to induce TNF-α production in these cells. After 6 hrs incubation with CT-1, a significantly increased up to 6.4-fold expression of TNF-α protein was observed in PBMC, but no significant changes were found in IL-2, IL-4, IL-5, IL-10 and IFN-γ expression. These data confirm that CT-1 can activate PBMC to produce cytokine, in which TNF-α is involved but not the cytokines of IL-2, IL-4, IL-5, IL-10 and IFN-γ.

In addition, given that TNF-α is primarily screted from monocytes and macrophages but not from lymphocytes, while IL-2, IL-4, IL-5, IL-10 and IFN-γ are produced primarily by lymphocytes (IL-2 is produced by helper T lymphocytes (Th cells), IL-4 by macrophages and Th2 cells, IL-5 by Th2 cells, IL-10 by monocytes, macrophages and Th2 cells, INF-γ by Th1 cells, Tc and natural killer cells), my results also indicate that this distinct cytokine production profile evoked by CT-1, which exhibits a unique up-regulated expression of TNF-α without IL-2, IL-4, IL-5, IL-10 and IFN-γ expression, suggests that the activation of PBMC might exclusively proceed in monocytes subset. This concern has been discussed later in this dissertation.

5.3 CT-1 induces the expression of TNF-α at both mRNA and protein levels in PBMC

The present study demonstrates that CT-1 alone is able to induce the expression of TNFα at both mRNA and protein level in a concentration- and time-dependent manner in PBMC from healthy volunteers. This effect is exclusively mediated via CT-1 receptor - gp130/LIFR-β heterodimer. My data indicate that CT-1, secreted from cardiac cells into the peripheral circulation in CHF, might activate PBMC to produce TNF-α and therefore CT-1 may be at least in part responsible for the elevated circulating levels of TNF-α in these patients.

Previous studies have shown that TNF-α was increased in serum of patients with CHF and correlated with the severity of heart failure, cachexia (Anker, Chua et al.

1997) and clinical outcome (Anker, Ponikowski et al. 1997). TNF-α may be involved in the progression of CHF because high levels of TNF-α can induce left ventricular dysfunction, ventricular remodelling, cardiomyopathy and pulmonary edema (Mann and Young 1994; Kelly and Smith 1997). Cultured human PBMC can synthesize and secrete TNF-α. In heart failure, both the heart itself as well as activated monocytes are able to secrete TNF-α (Torre-Amione, Kapadia et al. 1996; Vonhof, Brost et al. 1998). Furthermore, the capacity of PBMC of CHF patients to secrete TNF-α is increased compared with healthy control. My data are in good agreement with these former studies.

In my experiments both TNF-α mRNA expression and TNF-α protein production of PBMC showed a large standard variation. First one explanation for the large standard deviation may be a different genetic susceptibility of PBMC from different persons to stimuli (Hu, Luo et al. 2006). Second, I used the low basal mRNA or protein levels as basis of normalization explaining the large standard variation.

The fact that the increase of TNF-α mRNA expression after CT-1 application is much higher compared to the increase of protein in the supernatant may be explained methodically.

In addition, in my experiments I performed two methods (ELISA and flow cytometry) to determine the TNF-α protein production of PBMC in two different culture conditions of isolated purified PBMC cultures and human whole blood cultures respectively. In both of these two culture conditions an increasing expression of TNF-α protein induced by CT-1 was observed in PBMC, indicating that CT-1 induced TNF-α production from PBMC was independent of culture conditions and independent of protein determination methods.

As we can seen above, CT-1 can promote TNF-α mRNA expression and protein secretion from PBMC. It raised my another interest: What is TNF-α protein secretion dependent on? Is it dependent on mRNA synthesis or intracellular protein transport? To overcome these problems, I used actinomycin D to inhibite mRNA synthesis and brefeldin A to inhibite intracellular protein transport in PBMC 30 min before CT-1 incubation. I found that both inhibition of mRNA synthesis by actinomycin D and inhibition of intracellular protein transport by

brefeldin A were able to abolish CT-1 induced TNF-α protein production in the supernatant. These results demonstrated that CT-1 was responsible for new protein synthesis of TNF-α protein in PBMC, and that TNF-α protein was secreted into supernatant actively.

5.4 CT-1 activates PBMC to produce TNF-α: a new mechanism of upregulated synthesis of TNF-α in heart failure

It has been shown that activated monocytes are responsible for increased TNF-α serum concentrations in CHF. Monocytes may be activated by LPS or other ETX from the gut because the barrier function of the gut by cardial edema is disturbed and bacteria can easily translocate from the gut lumen into the blood (Anker, Egerer et al. 1997; Brunkhorst, Clark et al. 1999; Niebauer, Volk et al. 1999; Conraads, Jorens et al. 2004; Krack, Sharma et al. 2005; Torre-Amione 2005). Siminarly, I found an activation of PBMC and a significantly expression of TNF-α in these cells in the present study. However, unlike the previous studies, I found that PBMC were actived by CT-1 but not by LPS or other ETX, suggesting that CT-1 alone is sufficient to activate PBMC to produce TNF-α in these experimental conditions.

My data suggest at least in theory a new mechanism for TNF-α production of PBMC in heart failure. In response to ventricular stretch due to hemodynamic stress, cardiac cells significantly upregulate CT-1 secretion into the peripheral circulation (Jougasaki, Leskinen et al. 2003). Once CT-1 is released into peripheral circulation, it activates PBMC and is able to induce TNF-α production from these cells in the absence of other stimuli, cytokines or pathogens, e.g. LPS.

The mechanism presented herein might also explain why TNF-α may be still elevated in CHF even after edema were treated successfully with diuretics and the integrity of gut mucosa was restored. Furthermore, my data support a study from Petretta et al. that TNF-α is not produced by the failing heart or the gut in patients with mild to severe heart failure (Petretta, Condorelli et al. 2000).

5.5 Monocytes but not lymphocytes are the PBMC subsets involved in the CT-1 induced TNF-α production

Although TNF-α is thought to be produced primarily by monocytes and macrophages, there are several reports that demonstrate the ability of T lymphocytes to produce and release TNF-α (Fischer, Dohlsten et al. 1990; Akatsuka, Imanishi et al. 1994; Weill, Gay et al. 1996; Higuchi, Nagasawa et al. 1997; Yan, Yang et al. 1999). In the present study, intracellular TNF-α protein determination in $CD4^+$ and $CD8^+$ lymphocytes did not show any effects of CT-1 on TNF-α expression. In contrast, a concentration dependent significant increase of intracellular TNF-α after CT-1 application was found in monocytes. The present data show that CT-1 induced increasing expression of TNF-α in PBMC is not from the subpopulation of lymphocytes but from monocytes under these in vitro conditions.

Production of TNF-α after CT-1 stimulation in the subset of monocytes may reflect monocytes differentiation, because TNF-α has been described as an autocrine regulator of monocyte/macrophage differentiation (Witsell and Schook 1992). Monocytes activation and differentiation into macrophages will further exert their effects on inflammatory reaction and immunoregulation with lymphocytes.

Moreover, their role in heart failure is not restricted to inflammatory reaction. There is good evidence of the important roles of monocytes/macrophages in various cardiovascular disorders associated with heart failure. Monocytes have been involved in atherosclerosis, the pathophysiological process underlying coronary artery disease and subsequently of ischaemic cardiomyopathy (Ross 1999). Ischaemia-induced myocardial damage and subsequently myocardial remodelling and heart failure have also been associated with monocyte infiltration into the damaged myocardium (Roberts, Maclean et al. 1985; Swirski, Weissleder et al. 2009).

Therefore, as shown in my data the activation in monocytes induced by CT-1 means much more than a cytokine production, but suggests extensive effects of it on the pathophysiological process leading to heart failure.

5.6 CT-1 induces NFκB translocation into the nucleus in PBMC

5.6.1 NFκB

NFκB is a protein complex that controls the transcription of several genes. NFκB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low-density lipoprotein, and bacterial or viral antigens. NFκB plays a key role in regulating the immune response to infection. Incorrect regulation of NFκB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development.

There are five proteins in the mammalian NFκB family, as is shown in Table 2 (Nabel and Verma 1993).

Table 2 mammalian NFκB family members

Class	Protein	Aliases	Gene
I	NFκB1	p105 → p50	NFKB1
	NFκB2	p100 → p52	NFKB2
II	RelA	p65	RELA
	RelB		RELB
	c-Rel		REL

(Nabel and Verma 1993)

All proteins of the NFκB family share a Rel homology domain in their N-terminus. A subfamily of NFκB proteins, including RelA, RelB, and c-Rel, have a trans-activation domain in their C-termini. In contrast, the NFκB1 and NFκB2 proteins are synthesized as large precursors, p105, and p100, which undergo processing to generate the mature NFκB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective degradation of their C-terminal region containing ankyrin repeats. Whereas the generation of p52 from p100 is a tightly-regulated process, p50 is produced from constitutive processing of p105 (Karin and Ben-Neriah 2000; Senftleben, Cao et al. 2001) NFκB family members share structural homology with the retroviral oncoprotein v-Rel, resulting in their classification as NFκB/Rel proteins (Gilmore 2006) (Fig. 22).

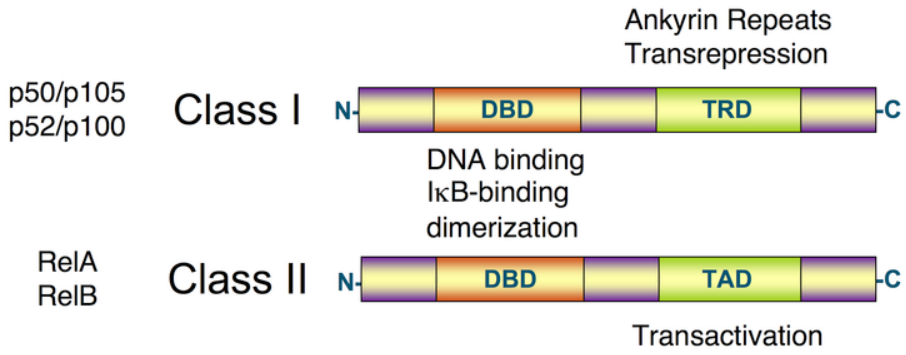

Figure 22. **Schematic diagram of** NFκB **protein structure**. There are two structural classes of NFκB proteins: class I (top) and class II (bottom). Both classes of proteins contain a N-terminal DNA-binding domain (DBD), which also serves as a dimerization interface to other NFκB transcription factors and, in addition, binds to the inhibitory IκBα protein. The C-terminus of class I proteins contains a number of ankyrin repeats and has transrepression activity. In contrast, the C-terminus of class II proteins has a transactivation function (Brasier 2006; Gilmore 2006; Perkins 2007).

5.6.2 Activation of NFκB

NFκB is important in regulating fast cellular responses because it belongs to the category of "rapid-acting" primary transcription factors, i.e., transcription factors that are present in cells in an inactive state and do not require new protein synthesis to be activated. This allows NFκB to be a first responder to harmful cellular stimuli. Known inducers of NFκB activity are highly variable and include reactive oxygen species, TNF-α, IL-1β, LPS, isoproterenol, cocaine, and ionizing radiation (Renard, Zachary et al. 1997; Basu, Rosenzweig et al. 1998; Takemoto, Yoshiyama et al. 1999; Chandel, Trzyna et al. 2000; Hargrave, Tiangco et al. 2003; Qin, Wilson et al. 2005; Fitzgerald, Meade et al. 2007).

Furthermore, many bacterial products and stimulation of a wide variety of cell-surface receptors lead to NFκB activation and rapid changes in gene expression (Gilmore 2006). The identification of Toll-like receptors (TLRs) as specific pattern recognition molecules and the finding that stimulation of TLRs leads to activation of NFκB improved our understanding of how different pathogens activate NFκB. For example, studies have identified TLR4 as the receptor for the LPS component of GNB (Doyle and O'Neill 2006). TLRs are key regulators of both innate and adaptive immune responses (Hayden, West et al. 2006).

Unlike RelA, RelB, and c-Rel, the p50 and p52 NFκB subunits do not contain transactivation domains in their C terminal halves. Nevertheless, the p50 and p52 NFκB members play critical roles in modulating the specificity of NFκB function. Although homodimers of p50 and p52 are, in general, repressors of κB site transcription, both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel (Li and Verma 2002). In addition, p50 and p52 homodimers also bind to the nuclear protein Bcl-3, and such complexes can function as transcriptional activators (Franzoso, Bours et al. 1992; Bours, Franzoso et al. 1993; Fujita, Nolan et al. 1993) (Fig. 23).

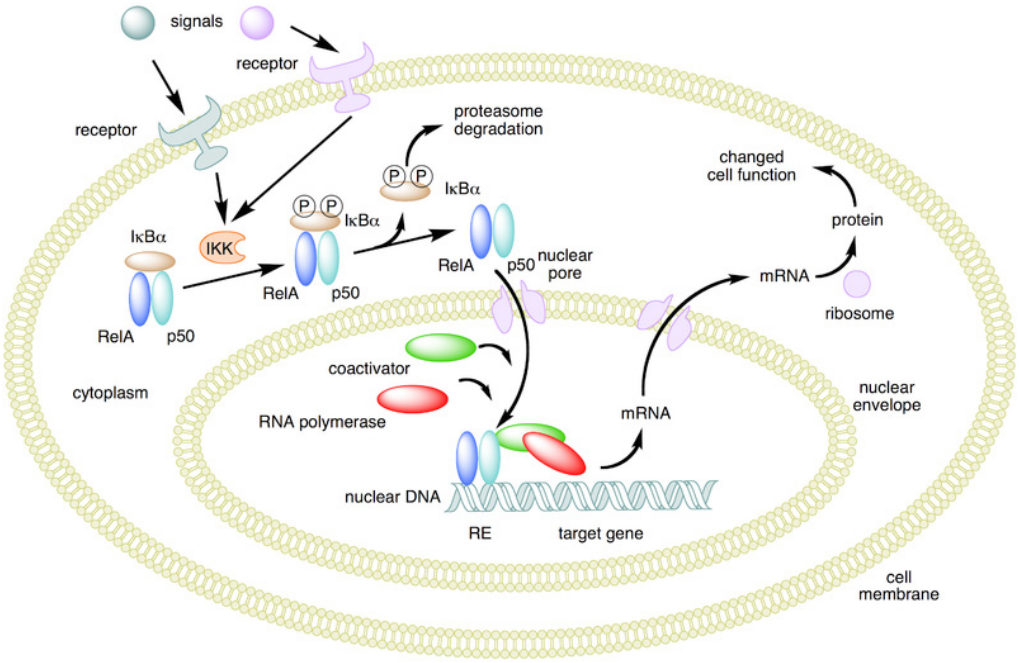

Figure 23. **Mechanism of NFκB action**. The NFκB heterodimer between Rel and p50 proteins is used as an example. While in an inactivated state, NFκB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IKK. IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NFκB, and eventual degradation of IκBα by the proteosome. The activated NFκB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements. The DNA/NFκB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function (Brasier 2006; Gilmore 2006; Perkins 2007).

5.6.3 Activation of NFκB in PBMC by CT-1

Previous studies have found an activation of the NFκB system in patients with heart failure. The myocardial tissue of patients with CHF of different etiologies exhibits overexpression of NFκB and of the genes that it regulates, such as TNF-α, NO, leukocyteadhesion molecules, and metalloproteinases (Valen, Yan et al. 2001). Peripheral blood cells were found an activation of the NFκB system in patients with CHF. Jankowska et al. reported an activation of the NFκB system in peripheral blood leukocytes in CHF patients measured by immuncytochemistry (Jankowska, von Haehling et al. 2005). Siednienko et al. found an augmented activation of NFκB activation in blood mononuclear cells using EMSA in patients with CHF compared with healthy controls (Siednienko, Jankowska et al. 2007). However, the exact pathway responsible for NFκB activation in CHF is still unknown and remains to be elucidated.

In the present study, CT-1 caused a concentration-dependent NFκB translocation into the nucleus in PBMC, indicating a CT-1 induced transcription factor activation. These data therefore suggest at least in theory a mechanism responsible for NFκB activation in CHF: In response to ventricular stretch due to hemodynamic stress, cardiac cells significantly upregulate CT-1 secretion into the peripheral circulation, which causes subsequent NFκB activation in PBMC from patients with CHF.

In addition, besides the known inducers of NFκB activity and its generally accepted receptors, I found that activation of NFκB can also be evoked by CT-1 stimulation via its gp130/LIFR-β receptor. These results indicate a new inducer of NFκB activity in PBMC and a new receptor for the NFκB signaling pathway.

5.7 CT-1 induced NFκB translocation into the nucleus in PBMC is mediated by IκB degradation

As shown in Figure 23, in unstimulated cells, the NFκB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs, which are proteins that contain multiple copies of a sequence called ankyrin repeats. By virtue of their ankyrin repeat domains, the IκB proteins mask the NLS of NFκB proteins and

keep them sequestered in an inactive state in the cytoplasm (Jacobs and Harrison 1998).

Activation of NFκB is initiated by the signal-induced degradation of IκB proteins. This occurs primarily via activation of a kinase called IKK. IKK is composed of a heterodimer of the catalytic IKK alpha and IKK beta subunits and a "master" regulatory protein termed NFκB essential modulator or IKK gamma. When activated by signals, usually coming from the outside of the cell, the IκB kinase phosphorylates two serine residues located in an IκB regulatory domain. When phosphorylated on these serines (e.g., serines 32 and 36 in human IκBα), the IκB inhibitor molecules are modified by a process called ubiquitination, which then leads them to be degraded by a cell structure called the proteasome (Lo, Basak et al. 2006; Basak, Kim et al. 2007) (Fig. 23).

With the degradation of IκB, the NFκB complex is then freed to enter the nucleus where it can 'turn on' the expression of specific genes. The activation of these genes by NFκB then leads to the given physiological response, for example, an inflammatory or immune response, a cell survival response, or cellular proliferation. NFκB turns on expression of its own repressor, IκBα. The newly synthesized IκBα then re-inhibits NFκB and, thus, forms an auto feedback loop, which results in oscillating levels of NFκB activity (Nelson, Ihekwaba et al. 2004) (Fig. 23).

In the present study, I found that CT-1 caused a decrease of IκB after 5 minutes of incubation, suggesting that CT-1 caused NFκB activation is medicated by IκB degradation. These data indicate that CT-1 induced NFκB activation is consistent with the other inducers via IκB degradation, which also supports previous finding in the mechanism of NFκB activation.

5.8 CT-1 induces TNF-α expression via NFκB pathway

5.8.1 Intracellular mechanisms leading to TNF-α production

In most cell types, the TNF-α gene is silenced (Beutler and Kruys 1995) but can be accessed in cell types that express the signaling mechanisms and transcriptional apparatus required for TNF-α production. Because monocytes

and macrophages are the main TNF-α source, mechanisms of TNF-α production have been studied almost exclusively in these cells. Although there are undoubtedly different cell types and different stimuli, the following signaling mechanisms describe what is presently known concerning LPS-stimulated macrophage TNF-α production (Sweet and Hume 1996).

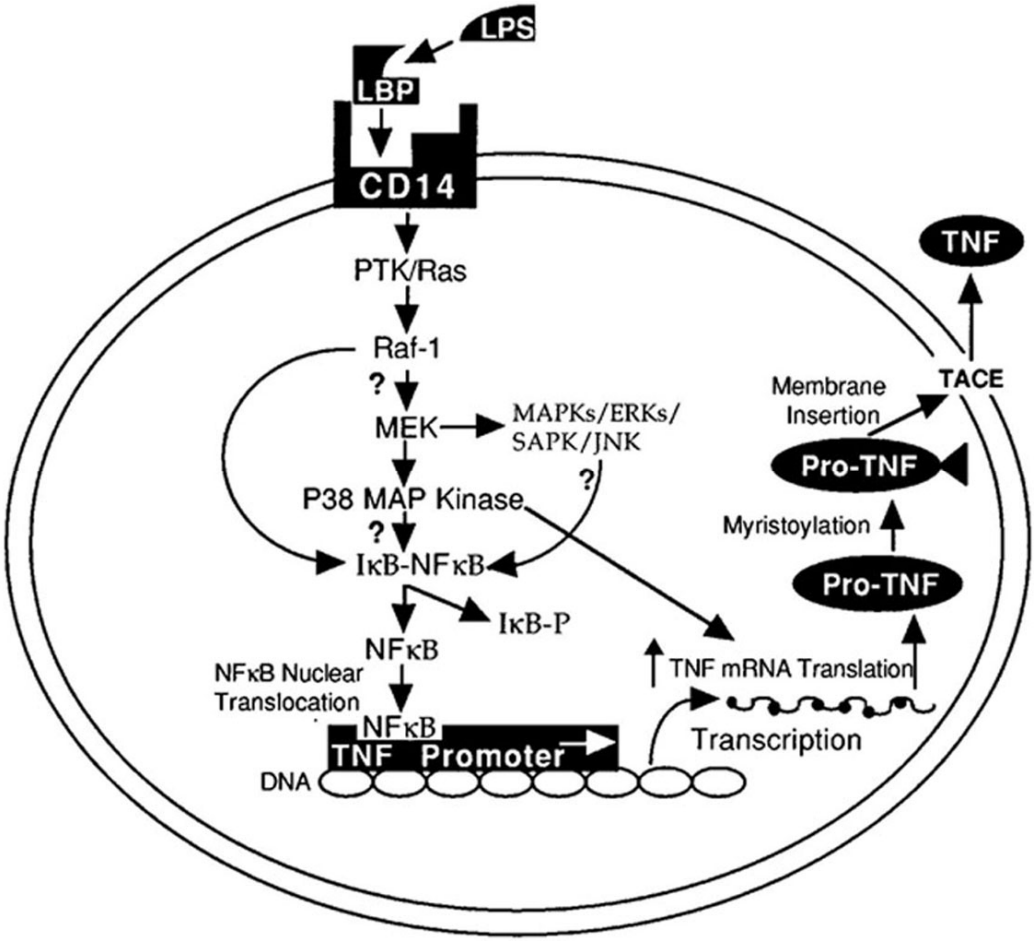

Figure 24. **Intracellular mechanisms that lead to TNF-α production.** LPS interaction with CD14 leads to rapid intracellular tyrosine phosphorylation of Ras [by phosphotyrosine kinase (PTK)], a process that initiates the protein kinase cascade, leading to TNF-α production. Ras activates Raf-1/ mitogenactivated protein kinase kinase, which activate members of the MAPK family of protein kinases, extracellular signal-related kinase, stress-activated protein kinase, and Jun nuclear kinase. The P38 MAPK appears to be an important MAPK in the cascade leading to TNF-α gene induction. NFkB is activated by IkB phosphorylation (which disengages its inhibitory subunit, IkB) and translocates to the nucleus to activate TNF-α promoters. Once translated to pro-TNF-α in the cytosol, myristoylation permits membrane insertion, where pro-TNF-α remains until it is cleaved to its mature form by tumor necrosis factor-alpha converting enzyme (TACE) (Meldrum 1998.).

LPS binds a variety of serum proteins that either positively or negatively influence the macrophage-mediated proinflammatory response (Ulevitch and Tobias 1995). Of these, LPS-binding protein (LBP) is the best characterized. LBP facilitates LPS binding to CD14 (Figs. 24). CD14 is the macrophage-specific surface receptor for LPS. Although not absolutely required for LPS-induced macrophage activation (at high doses LPS can bypass CD14 by triggering the p80 LPS receptor directly), LBP is required for LPS-CD14 interaction. Under normal circumstances, LPS-LBP interaction with CD14 is obligate for the intracellular signals that triggers LPS-induced TNF-α production.

As shown in Figure 24, LPS-LBP-CD14 interaction provokes rapid activation of protein tyrosine kinase (PTK) causing tyrosine phosphorylation of several intracellular protein kinases (Weinstein, Gold et al. 1991; Weinstein, Sanghera et al. 1992; Han, Lee et al. 1993; Weinstein, June et al. 1993; Shapira, Takashiba et al. 1994; Sanghera, Weinstein et al. 1996). PTK activates a pathway involving Ras/Raf-1/MEK/MAPKs/NFkB (Buscher, Hipskind et al. 1995; Derijard, Raingeaud et al. 1995; Levitzki 1995; Levitzki and Gazit 1995). Ras is an early target of activated PTK and is able to interact directly with Raf-1 (Howe, Leevers et al. 1992; Moodie, Willumsen et al. 1993; Van Aelst, Barr et al. 1993). Raf-1 is an important intermediate to MAPK activation (Howe, Leevers et al. 1992; Moodie, Willumsen et al. 1993; Van Aelst, Barr et al. 1993; Buscher, Hipskind et al. 1995). LPS resulted in PTK-dependent rapid phosphorylation and activation of Raf-1(Reimann, Buscher et al. 1994). Raf-1/MEK appears to activate members of the MAPK family of protein kinases; of these the P38 MAPK appears to be a pivotal MAPK in the cascade leading to TNF-α gene induction (Han, Lee et al. 1994; Lee, Laydon et al. 1994; Han, Richter et al. 1995; Shapiro and Dinarello 1995; Lee and Young 1996). In most cells, NFkB exists in a latent state, unable to induce gene transcription (Malinin, Boldin et al. 1997). In this state, NFkB is bound to its inhibitory proteins, IkB, which mask its nuclear localization site; however, after phosphorylation of IkB, the NFkB-IkB complex is disrupted and IkB is degraded (Li and Sedivy 1993; Trede, Tsytsykova et al. 1995; Sweet and Hume 1996). Once liberated from IkB, NFkB translocates from the cytoplasm to the nucleus, where it docks to DNA at one of four NFkB binding (TNF-α promoter) sites (Shakhov, Collart et al. 1990) and initiates the

transcription of TNF-α gene. IkB may be phosphorylated by MAPKs (Malinin, Boldin et al. 1997); however, it is noteworthy that Raf-1, a relatively upstream component of this pathway, is also capable of activating NFkB (Howe, Leevers et al. 1992; Li and Sedivy 1993). Redundant or ‘‘skip’’ activation sequences may be designed to ensure NFkB activation following LPS challenge. Once TNF-α gene transcription occurs, TNF-α mRNA is translated into the 26-kDa TNF-α precursor (pro-TNF-α) in the cytoplasm (McGeehan, Becherer et al. 1994; Mohler, Sleath et al. 1994; Dinarello and Margolis 1995). Myristoylation in the cytoplasm facilitates membrane insertion/association, where it is cleaved by TACE. The mature 17-kDa TNF-α is then released into the extracellular space. Thus LPS interaction with CD14 leads to rapid intracellular tyrosine phosphorylation of Ras, a process that initiates the protein kinase cascade leading to NFkB activation and TNF-α production.

5.8.2 CT-1 induces TNF-α expression via NFκB pathway

In the present study, CT-1 caused translocation of NFκB into the nucleus of PBMC, which was completely inhibited by parthenolide, an inhibitor of NFκB activation. The fact that parthenolide is able to inhibit CT-1 induced TNF-α expression in PBMC at both protein and mRNA level, suggesting that NFκB translocation is essential for CT-1 induced TNF-α expression. In addition, these data also confirm that CT-1 promoting TNF-α production is in good agreement with the above-mentioned signaling pathway of NFκB.

As described in **Section 5.8.1**, there are two pathways in the NFkB activation for TNF-α expression: Raf-1/MEK activates members of the MAPK family of protein kinases (i.e. P38 MAPK and ERKs) leading to IkB phosphorylation and subsequent activation of NFkB (Han, Lee et al. 1994; Lee, Laydon et al. 1994; Han, Richter et al. 1995; Shapiro and Dinarello 1995; Lee and Young 1996), or Raf-1 directly activates NFkB as ‘‘skip’’ activation sequences (Howe, Leevers et al. 1992; Li and Sedivy 1993). In the present study, neither SB203580 (inhibitor of P38 MAPK) nor PD98059 (inhibitor of ERK) are capable of interruption of CT-1-induced TNF-α expression in PBMC, suggesting that CT-1 might activate NFkB via Raf-1 directly as ‘‘skip’’ activation sequences but not via

Raf-1/MEK/MAPKs pathway.

5.9 Limitations

This study has several limitations. I only used inhibitor experiments to characterise the pathway involved. Furthermore I used a relative high parthenolide concentration. But within 3 hours, there is no cytotoxic effect as shown by O`Neill et al. (O'Neill, Barrett et al. 1987). I also used high CT-1 concentrations compared to concentrations reported in patients with CHF by Ng et al. (Ng, O'Brien et al. 2002). On the other hand a paper published in 2008 (Natal, Fortuno et al. 2008) reported serum CT-1 concentration in healthy controls and patients with metabolic syndrome of about 100 ng/ml. So far, serum concentration of CT-1 in healthy controls and patients are a matter of discussion. However, independent of reported CT-1 serum concentration the concentration of CT-1 should be much higher in the myocardium which is the source of CT-1 in CHF (Asai, Saito et al. 2000). Exact intramyocardial CT-1 concentrations are not determined so far, only mRNA and immunhistochemical studies showed increased expression of CT-1 in hearts of patients with CHF (Zolk, Ng et al. 2002).

6 Conclusions

In conclusion, this study showed that both subunits for forming the heterodimer receptor of gp130/LIFR-β for CT-1 action were expressed on PBMC. Via this receptor, CT-1 activated PBMC by evoking ERK and P38 MAKP signaling pathways, and promoting TNF-α expression. CT-1 significantly induced TNF-α production at both mRNA and protein levels in PBMC. Moreover, it has been shown that the subpopulation of monocytes but not lymphocytes were responsible for this effect. I further found out that CT-1 can induce NFκB translocation into the nucleus in PBMC, which was mediated by IκB degradation. Parthenolide (an inhibitor of NFκB activation) interrupted nuclear translocation of NFκB and completely inhibited the CT-1-induced TNF-α expression in PBMC, whereas SB203580 (an inhibitor of P38 MAPK activation) and PD98059 (an inhibitor of ERK activation) had no effects on them. These results indicate that NFκB nuclear translocation is essential for CT-1-induced TNF-α expression in PBMC and that CT-1 might activate NFkB via Raf-1 directly as ‘‘skip’’ activation sequences but not via Raf-1/MEK/MAPKs pathway.

These observations offer a new mechanism of activated PBMC, increased serum TNF-α concentration and active NFkB signaling pathway in CHF, which have significant implications for revealing a new mechanism of immune activation in CHF. Interestingly, in the present study LPS was not needed for the induction of TNF-α expression in PBMC. Moreover, this study may also elucidate a potential role of CT-1 in the pathophysiology of CHF and illustrate that heart is not only just a target of the inflammatory state in heart failure, but also play a role in the activation of inflammatory response by secreting CT-1 into the peripheral circulation, at least in part as a cause for initiating or promoting the inflammatory response in heart failure. In the light of these results, modulating CT-1 may be an interesting pharmacological target in the treatment of CHF.

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8.1 Publications

1. Wang Z, Huang Z, Lu G, Lin L, Ferrari M. Hypoxia during pregnancy in rats leads to early morphologic changes of atherosclerosis in adult offspring. Am J Physiol Heart Circ Physiol. 2009, 296(5): 1321-1328

2. Wang L, Cai R, Lu G, Huang Z, Wang Z. Hypoxia during pregnancy in rats leads to the changes of the cerebral white matter in adult offspring. Biochem Biophys Res Commun. 2010, 396(2): 445-450

3. Wang Z, Huang Z, Lu G, Su R. The role of intrauterine chronic hypoxia on blood pressure in rats offspring. Chinese Journal of Arteriosclerosis. 2010, 8: 617-620. Chinese

4. Wang Z, Huang Z, Lu G. The role of intrauterine chronic hypoxia on vascular endothelial function in rats offspring. Chinese Journal of Arteriosclerosis. 2010, 9: 696-700. Chinese

5. Chen Y, Lu G, Li B, Wang Z. Cerebral vascular resistance and left ventricular myocardial performance in fetuses with Ebstein's anomaly. Am J Perinatol. 2009, 26(4):253-258

6. Fritzenwanger M, Meusel K, Jung C, Franz M, Wang Z, Foerster M, Figulla HR. Cardiotrophin-1 induces tumor necrosis factor alpha synthesis in human peripheral blood mononuclear cells. Mediators Inflamm. 2009;2009:489802. Epub 2010 Mar 10.

Posters

1. Wang Z, Hutschenreuther J, Nowak G, Kaden T, Hentrich J, Figulla HR, Ferrari M. Cofactors of Paradox Reaction to Acetylsalicylic Acid in Patients with Coronary Artery Disease. Die 76. Jahrestagung, Deutsche Gesellschaft für Kardiologie - Herz- und Kreislaufforschung. 2010

2. Fritzenwanger M, Wang Z, Foerster M, Figulla HR. Cardiotrophin-1 induziert TNF-α in humanen Monozyten. Die 75. Jahrestagung, Deutsche Gesellschaft für Kardiologie - Herz- und Kreislaufforschung. 2009.

8.2 Awards

2009	Candidate for academic and technology leaders of health system in Fujian Province, China
2009	Promotionsstipendium from Interdisziplinäre Zentrum für Klinische Forschung (IZKF), Friedrich-Schiller-University, Jena, Germany
2007	Lindau Nobel Laureates Meeting, Germany
2006	DAAD-Scholarship from Germany
2006	Doctoral student Scholarship funded by China Scholarship Council (CSC)
2004~2007	Fuguang Scholarship for doctoral student funded by Provincial Government, China
2002	Excellent graduation thesis, China
1997~2002	The Union Scholarship annually, the Highest Scholarship annually, three-Excellent Student award annually, China

8.3 Curriculum vitae

Name: Zhenhua Wang
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Education:

11 / 2007 ~ Present,	PhD student, Universitätsklinikum Jena, Germany Klinik für Innere Medizin I (Kardiologie, Angiologie, Pneumologie, Internistische Intensivmedizin)
08 / 2006 ~ 07 / 2007,	Guest PhD student, Friedrich-Schiller-University, Jena, Germany (DAAD Scholarship, Sandwich Model of cooperative PhD program)
09 / 2002 ~ 07 / 2007,	PhD, Fujian Medical University, China (Internal Medicine)
09 / 1997 ~ 07 / 2002,	Bachelor of Medicine (equivalent to U.S. MD degree), Fujian Medical University, China (Medicine)

Work experience:

08 / 2002 ~ 10 / 2007,	Resident physician, 2nd Affiliated Hospital of Fujian Medical University, China
03 / 2004 ~ 07 / 2006,	Experimental research in PhD program (the Role of Intrauterine Chronic Hypoxia on the Development of Atherosclerosis in Rats Offspring and Its Mechanism), Molecular Pharmacology and Gene Therapy Lab of Fujian Medical University, China
09 / 2003 ~ 02 / 2004,	Clinical research involved clinical applied study on Anatomical M-mode Echocardiography, 2nd Affiliated Hospital of Fujian Medical University, China
01 / 2002 ~ 07 / 2002,	Experimental research for Bachelor's thesis, Center of Disease Control of Fujian Province, China (Molecular epidemiology research on Mycoplasma pneumoniae)
01 / 2000 ~ 02 / 2001,	Intern, 2nd Affiliated Hospital of Fujian Medical University, clinical practice rotation

Jena, den 1. Juli 2011

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8.4 Acknowledgments

I would like to thank Prof. Dr. Dr. Markus Ferrari and Dr. Michael Fritzenwanger for their enthusiasm and support. I am most grateful for their kind advice, unrivalled assistence and perfect supervision of my PhD work. I learnt a great deal from them. I would like to thank Ms. Annett Schmidt for her constant support and excellent technical assistance. I am thankful also to Dr. Martin Förster and Ms. Yvonne Schlenker for their help and kindness throughout the course of my PhD work. I am especially grateful for the time we worked together in Forschungszentrum Lobeda (FZL).

8.5 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Herr Prof. Dr. Dr. Markus Ferrari, Herr Dr. Michael Fritzenwanger,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den 1. Juli 2011

Zhenhua Wang